Universidade de Aveiro Departamento de Biologia
Ano 2017

DANIELA VELOSO DA CONCEIÇÃO SOARES CORREIA

EFEITOS DE LONGO PRAZO DA EXPOSIÇÃO EMBRIONÁRIA A CONTAMINANTES EM PEIXE-ZEBRA

LONG-TERM EFFECTS OF EMBRYONIC EXPOSURE TO CONTAMINANTS IN ZEBRAFISH

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro Departamento de Biologia
Ano 2017

DANIELA VELOSO DA CONCEIÇÃO SOARES CORREIA

EFEITOS DE LONGO PRAZO DA EXPOSIÇÃO EMBRIONÁRIA A CONTAMINANTES EM PEIXE-ZEBRA

LONG-TERM EFFECTS OF EMBRYONIC EXPOSURE TO CONTAMINANTS IN ZEBRAFISH

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, no ramo Toxicologia e Ecotoxicologia, realizada sob a orientação científica de Doutora Paula Inês Borralho Domingues, Estagiária de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro e co-orientação de Doutor Marcelino Miguel Oliveira, Investigador auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro.

O júri

Presidente

Professor Doutor Ulisses Manuel de Miranda Azeiteiro
Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro

Doutora Marta Sofia Soares Craveiro Alves Monteiro dos Santos
Investigadora de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro

Doutora Paula Inês Borralho Domingues
Investigadora de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro

Agradecimentos

Estando a chegar ao fim desta jornada, é tempo de expressar o meu agradecimento às pessoas que, de diferentes formas, me acompanharam e ajudaram na concretização deste trabalho.

Em primeiro lugar à minha orientadora Doutora Inês Domingues. Agradeço-te pela tua orientação, por toda a ajuda que me deste, em conjunto com muita dose de paciência e compreensão. Por teres estado sempre disponível quando precisei e por me motivares quando eu não conseguia ver resultados. És realmente a melhor orientadora que poderia ter encontrado e se tivesse que voltar a escolher, escolhia-te novamente, sem pensar duas vezes. És uma excelente pessoa e espero no futuro voltar a trabalhar contigo.

Ao Dr. Miguel Oliveira, ter aceitado a co-orientação desta dissertação bem como a ajuda prestada durante a realização da mesma.

À Ana Rita Almeida, que arranjava sempre um pouco do seu tempo para me ajudar. Obrigada por toda a paciência que tiveste em ensinar como se faziam diversas tarefas no lab e, sobretudo, pela companhia no lab e almoços.

À Niedja Santos pelos conselhos, companhia e amizade.

To Mariana Grădinaru, for all your help to time the endless videos of my experiences. Without you, it would have taken a lifetime. Thank you for your company, friendship and for teaching me some things in Romanian.

Às minhas colegas de casa e amigas, Sónia, Catarina, Cláudia e Soraia, agradeço todos os momentos inesquecíveis e malucos que tivemos. Fizeram com que estes dois anos em Aveiro se tornassem memoráveis e únicos.

Aos melhores amigos que podia ter, Sofia, Bárbara e Henrique, que estiveram sempre do meu lado, apoiando-me nos bons e nos maus momentos. Agradeço toda a vossa amizade, companhia, carinho, (muita) paciência e força dada e, apesar de estarem longe, estiveram sempre presentes nos momentos mais importantes.

E acima de tudo aos meus pais e irmão, porque sem eles nada disto seria possível. Por todo o amor, pelo incessante apoio, por todos os conselhos, por me apoiarem nas minhas escolhas, estando sempre do meu lado. Por tudo o que fazem por mim e, principalmente, por acreditarem sempre.

Palavras-chave

Danio rerio, exposição embrionária, concentrações sub-letais, carbamato, carbaryl, comportamento, fase adulta.

Resumo

Actualmente o meio aquático é contaminado por diversos poluentes ambientais, em quantidades de uma gama muito reduzida. Este tipo de cenário, designado de cenário de exposição ambiental realista, possui uma relevância importante porque os efeitos adversos que produz não são imediatamente perceptíveis, podendo apenas manifestar-se em fases de desenvolvimento mais tardias e também em parâmetros muito sensíveis como a nível comportamental. Neste trabalho pretendeu-se avaliar os efeitos de concentrações sub-letais de carbaryl, um insecticida carbamato, no comportamento de peixes zebra adultos, tendo estes sido expostos na fase embrionária ao carbamato. Após uma exposição aguda a várias concentrações de carbaryl, os organismos completaram o seu desenvolvimento até à fase adulta em meio limpo. Por conseguinte, realizaram-se diversos testes de comportamento, nomeadamente actividade locomotora e comportamento tigmotáctico, exploração natatória, comportamento alimentar, comportamento social (cardume) e comportamento de evitamento a predadores. No teste que visa a actividade locomotora e comportamento tigmotáctico, a resposta locomotora dos organismos pré-expostos ficou afectada, nadando uma menor distância, principalmente na presença de um estímulo de luz e os peixes expostos à concentração mais elevada mostraram uma diminuição do comportamento tigmotáctico nos períodos de escuro, não se observando uma habituação aos estímulos ao longo do tempo. Na exploração natatória os peixes expostos à concentração mais elevada possuíam uma menor tendência de exploração da superfície, permanecendo mais próximos do fundo. No teste da alimentação, as concentrações mais elevadas apresentavam um tempo mais longo na ingestão total do alimento. No teste de comportamento social, observou-se nos peixes expostos à concentração mais elevada uma maior tendência em permaneceram mais próximos do cardume. Contudo no teste de evitamento a predadores e na reprodução e desenvolvimento da F1, os nossos dados não nos permitem concluir se a pré-exposição a carbaryl afectou os peixes zebra. Todos estes resultados poderão ser uma evidência de uma disrupção em vários processos a nível fisiológico e bioquímico. De uma forma geral, os dados obtidos neste trabalho evidenciam que a exposição a carbaryl durante o desenvolvimento embrionário pode induzir a efeitos a longo prazo como as alterações comportamentais. Este cenário apresenta uma importante relevância ecológica que se deve ter em consideração em futuras avaliações de risco.

Keywords *Danio rerio*, embryonic exposure, sublethal concentrations, carbamate, carbaryl, behavior, adulthood..

Abstract Nowadays, the aquatic environment is contaminated by various environmental pollutants, in quantities of a very reduced range. This type of scenario, called a realistic environmental exposure scenario, has an important relevance because the adverse effects it produces are not immediately perceptible, may only manifest in later development stages and also in very sensitive parameters such as behavioral level. In this work we intend to evaluate the effects of sublethal concentrations of carbaryl, a carbamate insecticide, on the behavior of adult zebrafish, which were exposed in the embryonic stage to carbamate. After acute exposure to various concentrations of carbaryl, the organisms completed their development into adulthood in a clean environment. Therefore, several behavioral tests were carried out, namely locomotor activity and thigmotactic behavior, exploratory swimming, feeding behavior, social behavior (shoal) and avoidance behavior to predators. In the test aimed at locomotor activity and thigmotactic behavior, the locomotor response of the pre-exposed organisms was affected, swimming a smaller distance, mainly in the presence of a light stimulus and the fish exposed to the higher concentration showed a decrease of the thigmotactic behavior in the dark periods, with no habituation to the stimuli over time. In the exploratory swimming test, fish exposed to the highest concentration had a lower tendency to explore the surface, remaining closer to the bottom. The feeding test, the higher concentrations had a longer time in the total food intake. In the social behavior test, it was observed in the fish exposed to the higher concentration a greater tendency to remain closer to the shoal. However in the predator avoidance test and in the reproduction and development of F1, our data do not allow us to conclude if pre-exposure to carbaryl affected zebrafish. All of these results may be evidence of a disruption in various physiological and biochemical processes. In general, the data obtained in this study show that exposure to carbaryl during embryonic development may induce long-term effects such as behavioral changes. This scenario has an important ecological relevance that should be taken into account in future risk assessments.

Índice

List of Figures

Chapter 1: Introduction

Chapter 2: "Behavioral effects in adult zebrafish after developmental exposure to carbaryl"

Figure 7 – Effects of embryonic exposure to sublethal concentrations of carbaryl on adult zebrafish behavior. **A – Exploratory Swimming Test:** swimming behavior of adult zebrafish, with the three layers (bottom, middle and top) (mean value ± standard error (SE)); **B – Feeding Test:** feeding behavior of adult zebrafish. The results show the time spent on the first feeding action (black bars) and on food intake (grey bars), expressed as mean value ± SE; **C – Shoaling Test:** social behavior of adult zebrafish discriminated between the zone 1 – Close Proximity Zone, zone 2 – Neutral zone near the shoal and zone 3 – Neutral zone away from the shoal (mean value ± SE; **D - Predator Avoidance Test:** reaction of adult zebrafish to the presence of a predator, discriminated in Zone 1 – Close proximity zone (the area closest to the predator); Zone 2 - Neutral zone near the predator (in the middle of the aquarium) and Zone 3 – Neutral zone away from the predator (mean value ± SE).

Annex: Behavior Tests

Figure S1 – Illustrative diagram of the execution of locomotor activity and thigmotactic behavior.

Figure S2 – Protocol used for the analysis of thigmotactic behavior, with the division of the aquarium in 2 areas (**Area 1 –** Outer zone; **Area 2 –** Inner zone).

Figure S3 - Experimental assembly of the Exploratory Swimming test, with indication of the aquarium zones (Zone 1 **–** Bottom (blue), zone 2 **–** Middle (yellow) and zone 3 **–** Top (red)) (A) and print screen of the software ZebraLab®v3 Automated Behavioural Analysis used to aanalyse of the resulting videos (B).

Figure S4 – Experimental assembly of the Shoaling test, with three zones: **Zone 1 -** Close proximity zone (the area closest to the shoal) (yellow color), **Zone 2 -** Neutral zone near the shoal (in the middle of the aquarium) (res color) and **Zone 3 -** Neutral zone away from the shoal (green color).

List of Tables

Chapter 2: "Behavioral effects in adult zebrafish after developmental exposure to carbaryl"

Chapter 1

Introduction

1. Introduction

1.1. Environmental effects of insecticides

The progressive growth of large urban and industrial centers has led to a rapid deterioration in the water resources quality due to increased pollution of rivers, lakes and reservoirs (Américo et al., 2012). This situation is the result of the constant introduction, into the aquatic environment, of industrial, domestic and agricultural effluents containing numerous chemical compounds. The increased use of pesticides is one of the examples, having become a worldwide problem. Despite the legislation, pesticide use is affecting not only the environment but also the human population.

According to U.S. EPA (Environment Protection Agency), world pesticide amount used in 2011 and 2012 was approximately 2721554 tons (Fig.1) (Atwood & Paisley-Jones, 2017).

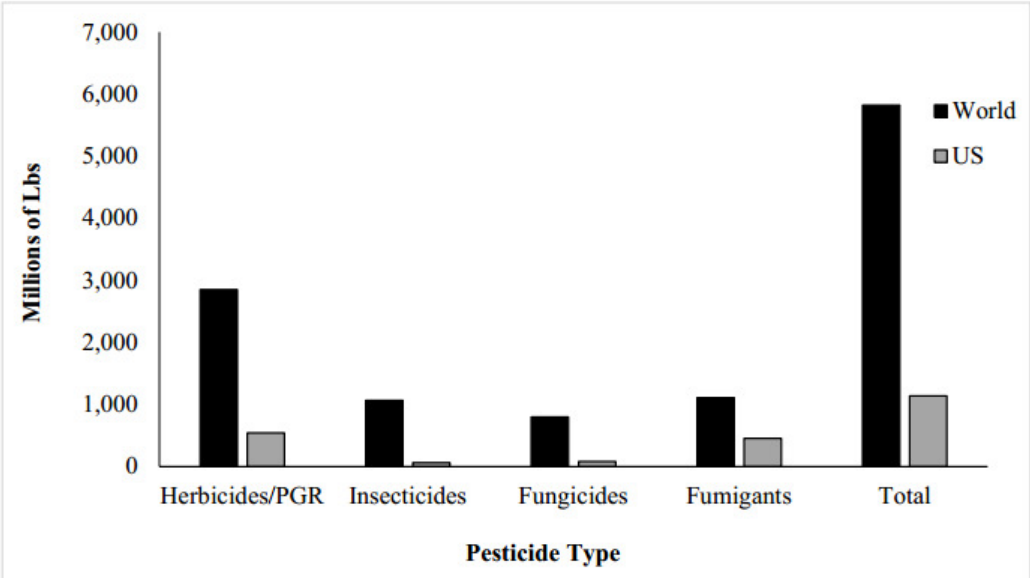

Figure 1 – World and U.S. amount of the various types of pesticides used (2012 estimate) (Atwood & Paisley-Jones, 2017).

Normally, an ideal pesticide should be lethal only to the targeted organisms. However, pesticides residues can reach several environmental compartments effecting soil, surface water, ground water, vegetation and non-targeted species. Pesticides that are soluble in water are easily transported into surface waters and those adsorbed into soil particles can be carried from application sites onto the sediment (Boran et al., 2007). Once there, they can harm plants and animals ranging from beneficial soil microorganisms and insects, nontarget plants, fish, birds, and other wildlife (Aktar et al., 2009).

Pesticides can be divided into herbicides, insecticides, fungicides, nematicides, fumigants and other miscellaneous conventional pesticides (Grube et al., 2011). Insecticides are generally the most toxic class of pesticides (Aktar et al., 2009). In 2012, world insecticide amount used was 18% of the total pesticides used, equivalent to 483075.8 kg approximately (Atwood & Paisley-Jones, 2017).

Carbaryl (1-naphthyl-N-methylcarbamate) is one of the most used carbamate insecticides, with agricultural and residential/ household applications as insecticides and fungicides (Fig. 2) (Boran et al., 2007; Lin et al., 2007). It is estimated that this contaminant is one of the active compounds found used in home and garden sectors, raging its quantity between 907.2 and 1814.4 tons in the United States of America, in 2012 (Atwood & Paisley-Jones, 2017). Carbamate insecticides are toxic to non-targeted wildlife such as fish and birds, which prove to be more sensitive than mammals to these compounds. Although carbamates do not have a high stability under aquatic conditions and have a short lifetime in the environment, bioaccumulation can occur in fish mainly due to the slow metabolism of these organisms (Boran et al., 2007).

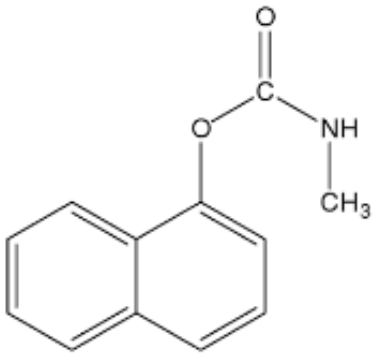

Figure 2 – Chemical structure of carbaryl (Mansour, 2008).

Carbaryl, like other carbamate substances, has a neurotoxic effect, acting as an acetylcholinesterase (AChE) inhibitor in the nervous system. AChE plays an important role in neuronal and muscle development and axonal growth (Schock et al., 2012). This enzyme is responsible for the conversion of acetylcholine, a neurotransmitter, into acetate and choline at the synapse and, when inhibited, leads to an accumulation of acetylcholine causing a consequential disruption of the nervous system (Boran et al., 2007; Bridges, 1997; Lin et al., 2007; Schock et al., 2012; Todd & Van Leeuwen, 2002). As a consequence, causes severe decrease in mobility, paralysis (at high concentrations) and even premature death (Boran et al., 2007; Bridges, 1997; Schock et al., 2012).

Carbaryl is also considered a substance with teratogenic properties, affecting physiological functions and hatching delay, increasing vulnerability to predation. Also it induces changes in fish embryos morphology like size and shape, defects in heart formation, with a development delay in cardiac looping and decreasing heart rate (Lin et al., 2007; Schock et al., 2012; Todd & Van Leeuwen, 2002).

Nowadays there are many studies about the carbaryl's effects in other aquatic organisms such us many species of frogs, salamanders and other fish species.

In plains leopard frog *Rana blairi*, tadpoles were exposed to three sublethal concentrations of this carbamate (3.5, 5.0 and 7.2 mg/L) and it was observed a negative effect in tadpoles swimming performance and spontaneous swimming activity. The swimming performance was severely damaged and the activity time decreases approximately 90% compared to the control group. Tadpoles had a lower growth rate, making them more vulnerable to predators, indirectly decreasing their survival chances. The sublethal concentrations tested also caused a reduction of sprint speed and distance travelled during sprint. With the increase of carbaryl concentration, the swim distance travelled was smaller. The effects previously mentioned can have a significant impact on predator's escape, affecting tadpoles survival, as well demographic structure of the population and its life cycle (Bridges, 1997).

In a study carried out in juvenile rainbow trout (*Oncorhynchus mykiss*) and guppies (*Poecilia reticulata*) was found that carbaryl had a more toxic effect on rainbow trout than on guppies, and this toxicity was greater with increasing concentration and exposure time to carbamate. It was observed that fish exposed to carbaryl suffered physiological and behavioral damages such as excessive mucus, rapid respiration rates, hyperactivity and

erratic movements. Both fish groups had loss of equilibrium and decreased swimming behavior and the highest mortality rate was recorded at the highest concentration (Boran et al., 2007).

In larval rainbow trout (*Oncorhynchus mykiss*) there was a decrease in cerebral cholinesterase (ChE) activity, which was partially inhibited by carbaryl exposure. With increasing concentration and exposure time, swimming speed also decreased, affecting escape from predators, feeding capacity, migration and even survival (Beauvais et al., 2001).

Tripathi & Singh (2002) performed a study with the freshwater snail *Lymnaea acuminate*, with the aim of analysing the impacts of sublethal concentrations of dimethoate and carbaryl (3.0, 6.0, 9.0 and 12.0 mg/L) in the metabolism of carbohydrate. They discovered that the organisms exposed to carbaryl had the levels of glycogen, pyruvate and lactate significantly altered, as well as lactic dehydrogenase (LDH) activity in hepatopancreas and ovotestis tissues. Following exposure, there was a reduction in glycogen and pyruvate levels and an increase in lactate levels in both hepatopancreas and ovotestis tissues. Also the LDH activity increased in both tissues. All of these changes may be due to the hypoxia phenomenon, induced by the pesticides used. The decrease of the glycogen and pyruvate occurred due to its high metabolism to obtain new energy to be able to diminish the conditions of hypoxia induced by pesticides used. Due to the occurrence of hypoxia, the oxygen levels decreased, leading organisms to perform anaerobiosis, increasing the amount of lactate produced. The conditions mentioned above also led to the increase of LDH, with the conversion of pyruvate to lactate (Tripathi & Singh, 2002).

Although the studies previously mentioned test the exposure to sublethal concentrations, even lower concentrations are found in the environment, not being detected. These do not cause immediately observable effects as happens in conventional exposure cases. For example, in the U.S.A., in urban areas, the lowest concentration of carbaryl detected was 0.0005 µg/L and the highest concentration was 33.5 µg/L, in agricultural areas (Mansour, 2008).

For a correct environment risk identification, the scientific community have to consider other more realistic scenarios as these “tests with more realistic exposure regimes provide a more accurate exposure–response model, where the pattern of exposure

(concentration vs time profile) is as similar as possible to that encountered in the field". (Peterson et al., 2001). As an example of these tests we have the exposure by pulses, exposure by mixtures and the embryonic exposure with evaluation of effects in adulthood.

Pulse exposure occurs due to surface runoff after rainfall, groundwater flow, drain flow, spray drifts or accidental spillage of pesticides applied to agricultural fields. (Azevedo-Pereira et al., 2011; Cold & Forbes, 2004; Pedersen et al., 2013; Peterson et al., 2001). This type of exposure can have a duration of minutes or hours, depending on the pesticide used (Cold & Forbes, 2004; Pedersen et al., 2013). In this way, aquatic ecosystems are affected with lethal or sublethal concentrations of chemicals, leading to a decrease in biodiversity (Azevedo-Pereira et al., 2011).

Exposure by pesticides mixture is also a very frequent scenario in the aquatic environment. These mixtures may result in synergistic, antagonistic and additive effects, affecting the organisms throughout their development (Hasenbein et al., 2015). In some cases, the combined effect of various pesticides may have a more pronounced negative impact than an isolated contaminant (Wang et al., 2017).

When embryonic exposure occurs, the effects from this may be various and can be identified in adulthood. These effects may have deleterious consequences, regardless of the level of chemical toxicity, provoking neurobehavioral effects with developmental exposure. In addition, exposure during embryonic development to carbamates could influence other mechanisms associated to behavior (Abreu-Villaça & Levin, 2017).

The occurrence of stressful events during embryonic stage may lead to the development of differential stress phenotypes in adulthood. This phenomenon is designate as developmental programming, remaining evolutionarily conserved. Modulation of developmental programming can affect other levels than growth and metabolism. It is increasingly evident that stressful events, induced by environmental factors, lead to the development of a range of phenotypes, from a single genotype. These different phenotypes are not immediately visible, having a long-term effect (Steenbergen et al., 2011).

All of these realistic exposure scenarios previously mentioned can lead to negative effects on several levels like, for example, changes in the social, feeding (Todd & Van Leeuwen, 2002) and swimming behavior (Beauvais et al., 2001; Boran et al., 2007), which can affect an entire specie and the community where they are inserted.

Thus, behavioral endpoints allow the evaluation of the sensitivity of organisms (Bridges, 1997) and detect the "hidden" impact of these sublethal concentrations.

An example of this, is the study carried out by Bailey et al. (2015) to observe the effects of ethanol on zebrafish behavior. After a brief exposure to sublethal concentrations of this organic substance in the embryonic stage and growth up to 2 months of age in a clean environment, behavioral tests were performed, such as Novel Tank dive Task, Tap Startle and habituation test and Spatial discrimination learning test.

The Novel tank dive Task aims to evaluated anxiety-like behavior by placing the zebrafish in a new environment. The second behavior test, tap startle and habituation test, consists in the evaluation of sensorimotor function and habituation learning using tap startle. Finally, the spatial discrimination learning test assess spatial learning and memory in zebrafish. In the novel tank dive task and in the tap startle and habituation test, they realized that the fish exposed to ethanol presented a greater hyperactivity, that is, there was a greater exploration of the aquarium compared to the control group. With these results, they concluded that embryo exposure may later influence zebrafish behavior, with severe changes in locomotion activity and exploration of the environment (Bailey et al., 2015).

Also in a study by Powers et al. (2011) using the exposure of zebrafish embryos to sublethal concentrations of silver, revealed a negative effect on the levels of the neurotransmitters dopamine (DA) and serotonin (5-hydroxytrypamine or 5HT). These play a determining role in anxiety and learning and, undergoing deregulation, can affect the behavior of zebrafish. In order to observe these effects, a behavioral test with 3 chambers was performed.

In the literature, other authors have also demonstrated the effects that contaminants can produce on behavior (Fernandes et al., 2015; Fernandes et al., 2014; Glazer et al., 2016; Levin et al., 2011; Roy et al., 2012; Truong et al., 2012; Vignet et al., 2014).

Considering the findings described above, in this work I intend to evaluate the effects of embryonic exposure to sublethal concentrations of carbaryl, a carbamate insecticide, in order to observe the consequences that this may cause on zebrafish behavior when they are in adulthood.

1.2. Zebrafish (*Danio rerio*)

The zebrafish is a small tropical fish, belonging to the class Actinipterrygii, order Cypriniformes and family Cyprinidae. Native of the rivers of South Asia, north-eastern India, Nepal and North of Bangladesh (Scholz et al., 2008; Spence et al., 2006; Spence et al., 2008), is commonly found in shallow lagoons and in irrigation canals, often linked to rice cultivation fields (Engeszer et al., 2007; Spence et al., 2006; Spence et al., 2008).

These organisms have a length between 3 to 5 cm (Wong, 2011), with a fusiform shape and laterally compress. The zebrafish is characterized by the presence of an incomplete line, in the lateral zone of the body, extending this until the pelvic fin. It is also observed the existence of five to seven dark blue longitudinal stripes, which extend from the operculum to the caudal fin (Spence et al., 2008). The males and females of this species have a similar coloration. However, females, in the breeding season, present a more rounded body shape, while males have a slimmer body shape (Fig. 3).

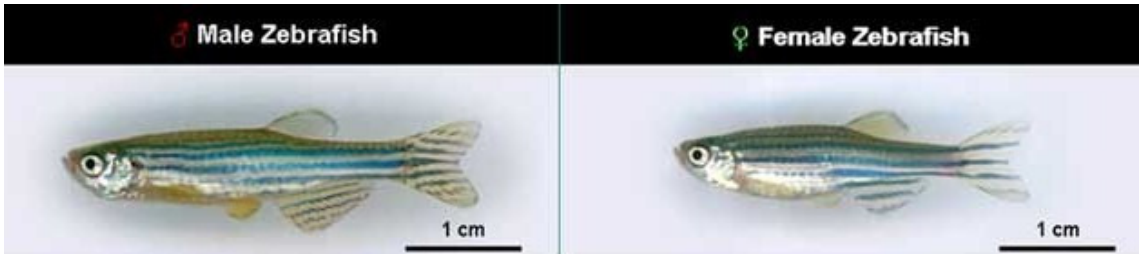

Figure 3 – Male and Female Zebrafish (*Danio rerio*) (Lab, 2014).

The zebrafish is an omnivore species, getting its food from the water column. The diet consists mainly of zooplankton, phytoplankton, filamentous algae and insects such as arachnids, mosquito larvae and invertebrate eggs (Engeszer et al., 2007; Spence et al., 2008). They have a very short life cycle and their reproductive maturity is reached approximately between 3 and 5-6 months old, depending if they are in their natural habitat or in laboratory conditions (Engeszer et al., 2007; Scholz et al., 2008; Spence et al., 2008). Fertilization is performed externally and a female can produce, in a single spawning, hundreds of eggs. These eggs have approximately 0.7 mm of dimension and a transparent

aspect, allowing the observation of different stages of development and their manipulation (Fig. 4) (Kimmel et al., 1995; Scholz et al., 2008; Spence et al., 2008). Due to its rapid embryonic development, the basic plan of the body is set to 24 hpf (hours post fertilization) and the hatching period occurs at 48 – 72 hpf (hours post fertilization). A complete consumption of yolk and organogenesis occurs at 5 dpf (days post fertilization), with larvae feeding externally (Scholz et al., 2008). After hatching, the larvae grow very quickly and begin to have a more active swimming behavior, allowing them to escape to predators and search for food (Kimmel et al., 1995; Scholz et al., 2008).

1.3. Zebrafish as a model for toxicology

Zebrafish has become an important model organism and is widely used for studies in several areas such as development biology, genetics, biomedical research (Spence et al., 2008), neurobiology and neurophysiology (Spence et al., 2008; Stewart et al., 2010), transgenic studies (Lele & Krone, 1996), pharmacology and ecotoxicology (Scholz et al., 2008). This vertebrate model gained an important position in the scientific investigation due to a wide set of characteristics like its small size, rapid development, high number of eggs produced by a single female, having these eggs a translucent appearance (Briggs, 2002; Scholz et al., 2008; Wong, 2011).

Its reduced size allows an easy maintenance in laboratory and reduced associated costs. The transparent chorion of the eggs provide the observation of different development stages and its genetic manipulation, studying cellular processes and development (Wong, 2011). The use of zebrafish embryos can be considered as an alternative to experiments with animals, since these organisms have a complex multicellular system which involve the interaction of various tissues and differentiations processes (Scholz et al., 2008).

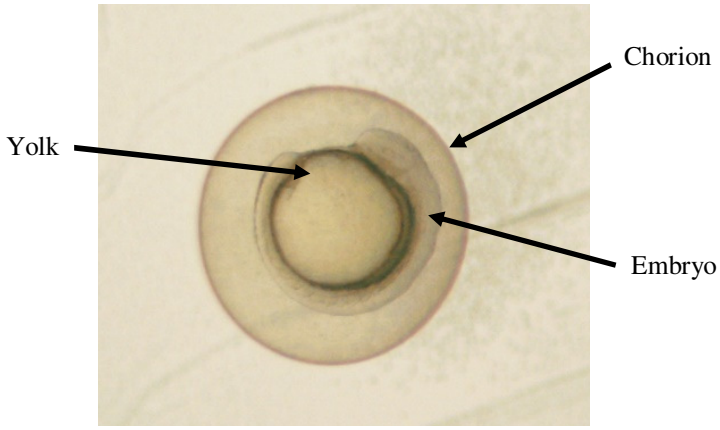

Figure 4 – Zebrafish embryo.

Being considered a good vertebrate model, comparisons can be made with other vertebrates like humans (Spence et al., 2008). The zebrafish genome is completely sequenced, sharing homologous sequences with the human genome and the amino acid sequences are evolutionarily conserved (de Esch et al., 2012; Postlethwait et al., 1998). In addition, it shares brain patterns, structure, functions of the neurochemical and behavioral systems and organization and functioning of its regulatory systems to stress events (Champagne et al., 2010). In this way, a more complete study of human diseases and the development (Scholz et al., 2008) and research of brain/neurobehavioral disorders (Champagne et al., 2010; Steenbergen et al., 2011; Stewart et al., 2010) is possible.

Currently, methodologies have been developed to evaluate the behavior of zebrafish, based on traditional behavior-based screening methodologies, used in rodent models. However this process is still at an early stage, depending on the characterization of behavioral tests, in order to better understand the responses to stress and anxiety. Given this, we can create new behavioral tests and valid behavioral tools (Blaser et al., 2010; Champagne et al., 2010; Steenbergen et al., 2011).

Two methodologies that were adapted to zebrafish were the open field and the light/ dark box test (Blaser et al., 2010; Champagne et al., 2010; Gerlai et al., 2000; Maximino et al., 2010). These tests are simple and painless, allowing an easy evaluation of the tendency of the organism to explore or submit a new environment, according to the averseness degree (Champagne et al., 2010; Steenbergen et al., 2011).

1.4. Aims

More and more the population has resorted to the use of pesticides for pests control and every day new chemical compounds are produced. Many of these compounds harm aquatic ecosystems and may have effects on life cycle and survival of organism there. The amounts of these compounds are usually very low (sublethal concentrations) and conventional ecotoxicity assays do not detect these effects on organisms; thus more relevant exposure scenarios are needed.

The main objective of this work is to evaluate the effects of embryonic exposure to sublethal concentrations of a contaminant, carbaryl, observing the consequences that these can produce on zebrafish behavior when they are in adulthood.

To reach this main objective, the work was divided into 4 phases:

1. Realization of an acute exposure of zebrafish embryos to sublethal concentrations of carbaryl;
2. Daily maintenance of these embryos in non-contaminated medium until adulthood;
3. Behavioral tests, in order to observe possible consequent effects of embryonic exposure to carbaryl, such as the Locomotion activity and thigmotactic behavior, Exploratory Swimming test, Feeding test, Shoaling test and Predator Avoidance test;
4. Reproduction and F1 embryonic development, to assess if embryonic exposure to carbaryl affects the quantity and quality of eggs and the embryo development of F1 generation.

1.5. Thesis Structure

- Chapter 1: Introduction;
- Chapter 2: "Behavioral effects in adult zebrafish after developmental exposure to carbaryl";
- Chapter 3: Conclusion and Future Perspectives.

References

Abreu-Villaça, Y., & Levin, E. D. (2017). Developmental neurotoxicity of succeeding generations of insecticides. *Environment International*, *99*, 55–77. https://doi.org/10.1016/j.envint.2016.11.019

Aktar, M. W., Sengupta, D., & Chowdhury, A. (2009). Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary Toxicology*, *2*(1), 1–12. https://doi.org/10.2478/v10102-009-0001-7

Américo, J. H. P., Messias, T. G., Torres, N. H., & Américo, G. H. P. (2012). Desreguladores endócrinos no ambiente e seus efeitos na biota e saúde humana. *Pesticidas: Revista de Ecotoxicologia E Meio Ambiente*, *22*, 17–34.

Atwood, D., & Paisley-Jones, C. (2017). Pesticides Industry Sales and Usage 2008-2012 Market Estimates. *U.S. Environmental Protection Agency*, (November).

Azevedo-Pereira, H. M. V. S., Lemos, M. F. L., & Soares, A. M. V. M. (2011). Behaviour and growth of Chironomus riparius Meigen (Diptera: Chironomidae) under imidacloprid pulse and constant exposure scenarios. *Water, Air, and Soil Pollution*, *219*(1–4), 215–224. https://doi.org/10.1007/s11270-010-0700-x

Bailey, J. M., Oliveri, A. N., Zhang, C., Frazier, J. M., Mackinnon, S., Cole, G. J., & Levin, E. D. (2015). Long-term behavioral impairment following acute embryonic ethanol exposure in zebrafish. *Neurotoxicology and Teratology*, *48*, 1–8. https://doi.org/10.1016/j.ntt.2015.01.005

Beauvais, S. L., Jones, S. B., Parris, J. T., Brewer, S. K., & Little, E. E. (2001). Cholinergic and behavioral neurotoxicity of carbaryl and cadmium to larval rainbow trout (Oncorhynchus mykiss). *Ecotoxicology and Environmental Safety*, *49*(1), 84–90. https://doi.org/10.1006/eesa.2000.2032

Blaser, R. E., Chadwick, L., & McGinnis, G. C. (2010). Behavioral measures of anxiety in zebrafish (Danio rerio). *Behavioural Brain Research*, *208*(1), 56–62. https://doi.org/10.1016/j.bbr.2009.11.009

Boran, M., Altinok, I., Capkin, E., Karacam, H., & Biçer, V. (2007). Acute toxicity of Carbaryl, Methiocarb, and Carbosulfan to the Rainbow Trout (Oncorhynchus mykiss) and Guppy (Poecilia reticulata). *Turkish Journal of Veterinary and Animal Sciences*, *31*(1), 39–45. Retrieved from

http://journals.tubitak.gov.tr/veterinary/abstract.htm?id=8715

Bridges, C. M. (1997). Tadpole swimming performance and activity affected by acute exposure to sublethal levels of Carbaryl. *Environmental Toxicology and Chemistry*, *16*(9), 1935–1939. https://doi.org/10.1002/etc.5620160924

Briggs, J. P. (2002). The zebrafish: a new model organism for integrative physiology. *AJP-Regulatory Integrative Comp Physiol*, *282*, 339–351. https://doi.org/10.1002/mus.10181

Champagne, D. L., Hoefnagels, C. C. M., de Kloet, R. E., & Richardson, M. K. (2010). Translating rodent behavioral repertoire to zebrafish (Danio rerio): Relevance for stress research. *Behavioural Brain Research*, *214*(2), 332–342. https://doi.org/http://doi.org/10.1016/j.bbr.2010.06.001

Cold, A., & Forbes, V. E. (2004). Consequences of a short pulse of pesticide exposure for survival and reproduction of Gammarus pulex. *Aquatic Toxicology*, *67*(3), 287–299. https://doi.org/10.1016/j.aquatox.2004.01.015

de Esch, C., Slieker, R., Wolterbeek, A., Woutersen, R., & de Groot, D. (2012). Zebrafish as potential model for developmental neurotoxicity testing. A mini review. *Neurotoxicology and Teratology*, *34*(6), 545–553. https://doi.org/10.1016/j.ntt.2012.08.006

Engeszer, R. E., Patterson, L. B., Rao, A. A., & Parichy, D. M. (2007). Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish*, *4*(1), 21–40. https://doi.org/10.1089/zeb.2006.9997

Fernandes, Y., Rampersad, M., & Gerlai, R. (2015). Impairment of social behaviour persists two years after embryonic alcohol exposure in zebrafish: A model of fetal alcohol spectrum disorders. *Behavioural Brain Research*, *292*, 102–108. https://doi.org/10.1016/j.bbr.2015.05.060

Fernandes, Y., Tran, S., Abraham, E., & Gerlai, R. (2014). Embryonic alcohol exposure impairs associative learning performance in adult zebrafish. *Behavioural Brain Research*, *265*, 181–187. https://doi.org/10.1016/j.bbr.2014.02.035

Gerlai, R., Lahav, M., Guo, S., & Rosenthal, A. (2000). Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. *Pharmacology Biochemistry and Behavior*, *67*(4), 773–782. https://doi.org/10.1016/S0091-3057(00)00422-6

Glazer, L., Hahn, M. E., & Aluru, N. (2016). Delayed effects of developmental exposure to low levels of the aryl hydrocarbon receptor agonist 3,3',4,4',5-pentachlorobiphenyl (PCB126) on adult zebrafish behavior. *NeuroToxicology*, *52*, 134–143. https://doi.org/10.1016/j.neuro.2015.11.012

Grube, A., Donaldson, D., Kiely, T., & Wu, L. (2011). Pesticides Industry Sales and Usage: 2006 and 2007 Market Estimates. *U.S. Environmental Protection Agency*, 1–41. https://doi.org/https://www.epa.gov/sites/production/files/2015-10/documents/market_estimates2007.pdf

Hasenbein, S., Lawler, S. P., Geist, J., & Connon, R. E. (2015). The use of growth and behavioral endpoints to assess the effects of pesticide mixtures upon aquatic organisms. *Ecotoxicology*, *24*(4), 746–759. https://doi.org/10.1007/s10646-015-1420-1

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics : An Official Public*, *203*(3), 253–310. https://doi.org/10.1002/aja.1002030302

Lab, M. (2014). Collecting Zebrafish Eggs. Retrieved March 22, 2017, from https://wiki.med.harvard.edu/SysBio/Megason/CollectingEggs

Lele, Z., & Krone, P. (1996). The zebrafish as a model system in developmental, toxicological and transgenic research. *Biotechnology Advances*. Retrieved from http://www.sciencedirect.com/science/article/pii/0734975096000043

Levin, E. D., Sledge, D., Roach, S., Petro, A., Donerly, S., & Linney, E. (2011). Persistent behavioral impairment caused by embryonic methylphenidate exposure in zebrafish. *Neurotoxicology and Teratology*, *33*(6), 668–673. https://doi.org/10.1016/j.ntt.2011.06.004

Lin, C. C., Hui, M. N. Y., & Cheng, S. H. (2007). Toxicity and cardiac effects of carbaryl in early developing zebrafish (Danio rerio) embryos. *Toxicology and Applied Pharmacology*, *222*(2), 159–168. https://doi.org/10.1016/j.taap.2007.04.013

Mansour, S. A. (2008). *Reviews of Environmental Contamination and Toxicology, Vol. 196*. (D. M.Whitacre, H. N. Nigg, & D. R. Doerge, Eds.). Springer. https://doi.org/10.1007/978-387-78444-1

Maximino, C., de Brito, T. M., da Silva Batista, A. W., Herculano, A. M., Morato, S., & Gouveia, A. (2010). Measuring anxiety in zebrafish: A critical review. *Behavioural*

Brain Research, *214*(2), 157–171. https://doi.org/10.1016/j.bbr.2010.05.031

Pedersen, S., Palmqvist, A., Thorbek, P., Hamer, M., & Forbes, V. (2013). Pairing behavior and reproduction in Hyalella azteca as sensitive endpoints for detecting long-term consequences of pesticide pulses. *Aquatic Toxicology*, *144–145*, 59–65. https://doi.org/10.1016/j.aquatox.2013.09.027

Peterson, J. L., Jepson, P. C., & Jenkins, J. J. (2001). Effect of varying pesticide exposure duration and concentration on the toxicity of carbaryl to two field-collected stream invertebrates, Calineuria californica (Plecoptera: Perlidae) and Cinygma sp (Ephemeroptera: Heptageniidae). *Environmental Toxicology and Chemistry / SETAC*, *20*(10), 2215–2223. https://doi.org/10.1897/1551-5028(2001)020<2215:EOVPED>2.0.CO;2

Postlethwait, J. H., Yan, Y. L., Gates, M. A., Horne, S., Amores, A., Brownlie, A., … Talbot, W. S. (1998). Vertebrate genome evolution and the zebrafish gene map. *Nature Genetics*, *18*(4), 345–349. https://doi.org/10.1038/ng0498-345

Powers, C. M., Levin, E. D., Seidler, F. J., & Slotkin, T. A. (2011). Silver exposure in developing zebrafish produces persistent synaptic and behavioral changes. *Neurotoxicology and Teratology*, *33*(2), 329–332. https://doi.org/10.1016/j.ntt.2010.10.006

Roy, N. M., Arpie, B., Lugo, J., Linney, E., Levin, E. D., & Cerutti, D. (2012). Brief embryonic strychnine exposure in zebrafish causes long-term adult behavioral impairment with indications of embryonic synaptic changes. *Neurotoxicology and Teratology*, *34*(6), 587–591. https://doi.org/10.1016/j.ntt.2012.08.001

Schock, E. N., Ford, W. C., Midgley, K. J., Fader, J. G., Giavasis, M. N., & McWhorter, M. L. (2012). The effects of Carbaryl on the development of zebrafish (Danio rerio) embryos. *Scientific Reports*, *9*(4), 169–78. https://doi.org/10.1089/zeb.2012.0747

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., & Voelker, D. (2008). The zebrafish embryo model in environmental risk assessment - Applications beyond acute toxicity testing. *Environmental Science and Pollution Research*, *15*(5), 394–404. https://doi.org/10.1007/s11356-008-0018-z

Spence, R., Fatema, M. K., Reichard, M., Huq, K. A., Wahab, M. A., Ahmed, Z. F., & Smith, C. (2006). The distribution and habitat preferences of the zebrafish in Bangladesh. *Journal of Fish Biology*, *69*(5), 1435–1448.

https://doi.org/10.1111/j.1095-8649.2006.01206.x

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). *The behaviour and ecology of the zebrafish , Danio rerio*. *Biological reviews of the Cambridge Philosophical Society*. https://doi.org/10.1111/j.1748-5827.2007.00030.x

Steenbergen, P. J., Richardson, M. K., & Champagne, D. L. (2011). The use of the zebrafish model in stress research. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *35*(6), 1432–1451. https://doi.org/10.1016/j.pnpbp.2010.10.010

Stewart, A., Kadri, F., DiLeo, J., Chung, K. M., Cachat, J., Goodspeed, J., … Kalueff, A. V. (2010). The Developing Utility of Zebrafish in Modeling Neurobehavioral Disorders. *Berkeley Planning Journal*, *23*(1), 104–120. https://doi.org/10.5811/westjem.2011.5.6700

Todd, N. E., & Van Leeuwen, M. (2002). Effects of Sevin (carbaryl insecticide) on early life stages of zebrafish (Danio rerio). *Ecotoxicology and Environmental Safety*, *53*(2), 267–272. https://doi.org/10.1006/eesa.2002.2231

Tripathi, P. K., & Singh, A. (2002). Toxic effects of dimethoate and carbaryl pesticides on reproduction and related enzymes of the freshwater snail Lymnaea acuminata. *Bulletin of Environmental Contamination and Toxicology*, *71*(3), 606–611. https://doi.org/10.1007/s00128-003-8873-2

Truong, L., Saili, K. S., Miller, J. M., Hutchison, J. E., & Tanguay, R. L. (2012). Persistent Adult Zebrafish Behavioral Deficits Results from Acute Embryonic Exposure to Gold Nanoparticles. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *155*(2), 130–134. https://doi.org/10.1016/j.pestbp.2011.02.012.Investigations

Vignet, C., Devier, M. H., Le Menach, K., Lyphout, L., Potier, J., Cachot, J., … Cousin, X. (2014). Long-term disruption of growth, reproduction, and behavior after embryonic exposure of zebrafish to PAH-spiked sediment. *Environmental Science and Pollution Research*, *21*(24), 13877–13887. https://doi.org/10.1007/s11356-014-2585-5

Wang, Y., Yang, G., Dai, D., Xu, Z., Cai, L., Wang, Q., & Yu, Y. (2017). Individual and mixture effects of five agricultural pesticides on zebrafish (Danio rerio) larvae. *Environmental Science and Pollution Research*, *24*(5), 4528–4536. https://doi.org/10.1007/s11356-016-8205-9

Wong, D. C.-M. (2011). *Conditioned place avoidance of Zebrafish (Danio rerio) to three*

chemicals used for euthanasia. University of British Columbia.

Chapter 2

Behavioral effects in adult zebrafish after developmental exposure to carbaryl

Behavioral effects in adult zebrafish after developmental exposure to carbaryl

(To be submitted to *NeuroToxicology*)

Daniela Correia[1], Ana Rita Almeida[1], Joana Santos[1], Vladimir Zlabek[2], Marcelino Miguel Oliveira[1] and Inês Domingues[1]

1 Department of Biology & CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

2 University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, Vodnany 389 25, Czech Republic

ABSTRACT

In recent years, there is a growing concern to correctly assess the risk of low environmental concentrations of contaminants (e.g. pesticides) to aquatic life. Pesticides can be found in the ng/L and µg/L range and, very often, observable effects to non-target organisms like fish, may not be perceptible immediately and long term effects can be underestimated. Hence, our work intends to evaluate the effect of an embryonic exposure to carbaryl in adult fish behavior. Zebrafish (*Danio rerio*) embryos were exposed to sublethal concentrations of carbaryl (0.01, 0.1 and 1.0 mg/L) plus a control for 4 days and then kept in standard cultivation conditions until adulthood. A battery of behavioral tests was then performed to assess anxiety-like behavior (locomotor activity, thigmotactic behavior and exploratory swimming test), social behavior (shoaling test), antipredatory behavior (predator avoidance test) plus a feeding test. Our data showed that zebrafish pre-exposed to 1.0 mg/L of carbaryl had lower swimming activity (distance moved) than non-exposed fish and presented reduced thigmotaxis during the dark stimulus when compared to light, suggesting repression of anxiety-like behavior. Other trends, although not statistically significant were considered important due to their consistency. This is the case of social behavior that seemed to be increased and exploratory and feeding behaviors that seem to be decreased. Our results show that developmental exposure to carbaryl has long lasting effects on adult behavior highlighting the importance of the environmental

conditions during early life stages of organisms. In a context of ecological risk assessment, this work shows that relevant scenarios including long term effects measured with sensitive and informative parameters such as behavior have to be considered for an accurate risk evaluation

Keywords: Embryonic exposure, carbamate, sublethal concentrations, adulthood, locomotion.

1. Introduction

The widespread use of pesticides and their environmental risks have driven in the last years to a huge amount of scientific studies accounting for their effects in non-target aquatic and terrestrial organisms. Many studies, however, may fail to accurately assess real effects on organisms because unrealistic exposure designs are used in the laboratory. Conventional acute and chronic testing has evolved as other exposure scenarios showed to be significant, such as life cycle assessments, mixture testing, pulse exposures or, with the advent of epigenetics, transgenerational assessment. The study of relevant exposure scenarios that better represent what happens in the environment is now a priority to achieve more accurate risk assessment strategies (Fleeger et al., 2003). For instance, recent evidences show that some phenotypic alterations due to chemical exposure may be observed in non-target organisms long after exposure has ceased. Implications of such effects are challenging for regulators, since "postponed" toxicity is rarely considered in environmental risk assessment. This scenario is particularly relevant for exposures in early life stages of organisms (e.g. embryo development), where effects are only manifested in the adulthood. "Early life stage stress events" are known to modulate the development programming of organisms with unpredictable long term effects (Steenbergen et al., 2011). Behavioral disruptions are among the most important long term effects observed and are particular relevant due to direct links to ecological important functions. For instance Bailey et al. (Bailey et al., 2015) exposed zebrafish (*Danio rerio*) embryos to several sub-lethal concentrations of ethanol. After being raised in a clean environment up to 2 months of age,

fish that have been exposed showed a hyperactive behavior when compared to the control. Similarly, Powers et al. (2011) also used zebrafish to evaluate long-term neurochemical and behavioral effects of an embryonic exposure to silver (Ag^+). In the adulthood zebrafish showed an increase in dopamine and serotonin levels, which play an important role in anxiety, sensorimotor integration and reward with consequent behavioral disruption (locomotion and avoidance behavior).

Behavior is being increasingly used to assess long term effects of early life stage stress events and shows to be an high sensitive endpoint for neuronal and endocrine disruption (Andrade et al., 2015; Klüver et al., 2015).

Recently, behavioral assessment methodologies have been developed for zebrafish (based on the traditional rodent behavior methodologies) (Champagne et al., 2010). These methodologies generally consist in exposing the fish to a mildly stressful situation eliciting typical innate fear or anxiety-like behaviors, which can be assessed by using the exploratory capacity of the fish and measuring behavioral components such as avoidance, freezing, erratic swimming, hyperactivity, shoaling, among others (Maximino et al., 2010). Since automated tracking systems are available in the market allowing fast and accurate analysis of zebrafish locomotor behavior, research in this area is growing, including the description of behavioral phenotypes and test validation. Behavioral endpoints allow the establishment of direct links to effects at population and community levels such as reproduction, capture of prey and escape of possible predators which are crucial for the maintenance of the population fitness.

In this work, the pesticide carbaryl was chosen as a model compound to assess behavioral disruption in adult zebrafish using an ecological relevant exposure scenario. Carbaryl is a widely used insecticide, belonging to the carbamates class (Bouchard et al., 2008; Branch and Jacqz, 1986; De Jesús Andino et al., 2017). Due to its toxicity, it can reach non-target species such as birds, fish and amphibians (De Jesús Andino et al., 2017). Carbaryl is considered as an acetylcholinesterase inhibitor (Mahajan et al., 2007; Muthaiah et al., 2016; Toumi et al., 2016), acting by blocking the degradation of the neurotransmitter acetylcholine into acetate and choline. This leads to an increase of acetylcholine in the synaptic cleft, causing adverse effects such as neurotoxicity, convulsions, paralysis and, in extreme cases, death (Branch and Jacqz, 1986; Çakıcı, 2016; Jeon et al., 2013; Mahajan et al., 2007; Muthaiah et al., 2016).

Thus, the main objective of the present study was to assess the long term effects of a developmental (embryonic) exposure to sublethal concentrations of carbaryl. This will be achieved by exposing zebrafish embryos for 96 hours and then raise them in standard (clean) conditions until adulthood where they will be tested for behavioral disruption. A battery of behavioral tests will then be used including the Locomotor activity test, the Exploratory Swimming test, the Feeding test, the Shoaling test and the Predator Avoidance test.

2. Materials and Methods

2.1. Test chemicals and test solutions preparation

The pesticide Carbaryl (1-Naphthyl-N-methylcarbamate) and an isotope-labelled internal standard (IS) of diclofenac ($^{13}C_6$) were purchased from Sigma-Aldrich and were used in this work.

A stock solution (2 mg/L) of carbaryl was done by dissolving the compound in culture water over the previous night. The concentrations used (0 (control), 0.01, 0.1 and 1.0 mg/L) (Andrade, 2015) were prepared from the stock solution by successive dilutions with culture water. The stock solution was wrapped in aluminum foil to avoid photodegradation. The renovation of test solutions was performed daily to ensure exposure concentrations. At the beginning of the assay 15 mL of each test solution were sampled in falcon tubes and stored at -20ºC for further chemicals analysis.

2.2. Chemical analysis

Stock solutions for chemical analysis were prepared in methanol at a concentration of 1 mg/mL. A spiking mixture of each was prepared by diluting the stock solution with methanol to a final concentration of 1 µg/mL. All of the stock and spiking solutions were

stored at −20°C. LC–MS grade acetonitrile (LiChrosolv Hypergrade) was obtained from Merck (Darmstadt, Germany). Formic acid was purchased from Labicom (Olomouc, Czech Republic) and was used to acidify the mobile phases. Ultrapure water was prepared via an Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea). The chemical analysis was performed at Laboratory of Environmental Chemistry and Biochemistry, University of South Bohemia in České Budějovice, Czech Republic. Briefly, 1 mL of thawed water samples were filtered through a syringe filter (0.45 mm, regenerated cellulose, Labicom, Olomouc, Czech Republic) and 10 ng of IS was added to the samples. Highly concentrated samples were d0iluted according to validated analytical method.

TSQ Quantiva triple-stage quadrupole mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA) coupled with Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and HTSXT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used to separate and detect the target analytes. An analytical Hypersil GOLD aQ column (50 mm length, 2.1 mm i.d., 5-µm particles; Thermo Fisher Scientific) was used to chromatographically separate the target analytes. A heated electrospray ionization (HESI-II) was applied in order to ionize the target compounds.

The method was validated in the concentration range of 0.001-1 mg/L and showed good linearity (R^2=0.994). Carbaryl recovery for water samples was 101±15% (triplicate analysis). Average limit of quantification was 0.007 mg/L. Matrix-matched standard response was used as factors for correcting the response derived from the calibration curve. The chromatographic gradient, other method parameters and results are presented in Annex - Table S1-3.

2.3. Test organisms and eggs collection

Zebrafish were kept in a ZebTEC (Tecniplast) recirculating system at University of Aveiro, Portugal. Culture water was obtained through reverse osmosis and activated carbon filtration of tap water, complemented with 0.34 mg/L salt (Instant Ocean Synthetic Sea Salt, Spectrum Brands), and automatically adjusted for pH and conductivity. Water temperature was 26±1 ºC, conductivity 800±50 µS, pH 7.5±0.5, and dissolved oxygen

equal or above 95% (7.6 mg/L) saturation. A 12:12-h light:dark photoperiod was maintained. The adult fish were fed twice a day with commercially available artificial diet (ZM-400 fish food; Zebrafish Management). Eggs were obtained as described in Andrade et al (2017).

2.4. Embryonic exposure

The assay was based on OECD guideline on Fish Embryo Toxicity Test (OECD, 2013). The embryos were exposed in petri dishes, with three replicates per treatment, each containing 130 embryos (3h post fertilization (hpf)). The concentrations chosen are sublethal and do not cause any observable developmental effect (anomalies or delays) during the 96 hours of the exposure. Embryos and larvae were observed daily for 96 hours. After this period, the larvae were transferred to a clean environment (culture water) in a larger aquarium and were maintained under culture conditions, in a static system. Density and feeding of the organisms were adjusted according to their growth until adulthood, with mortality being recorded over time. A battery of behavioral tests was then performed.

2.5. Behavior Tests

In all behavioral tests performed in this study fish to be tested were randomly selected and tested among the different concentrations to avoid bias. During the Exploratory Swimming test, Shoaling test and Predator Avoidance test, no people were allowed in the test room during the recordings, in order to reduce possible interferences (e.g. noise).

2.5.1. Locomotor activity and thigmotactic behavior

Locomotor activity was evaluated in ten fish per concentration. Fish were individually transferred to a rectangular container (9.4 cm wide, 4.6 cm high, 14.1 cm length and 1.6 cm water depth). The video tracking setting of the Viewpoint Zebrabox (Viewpoint Life Science, Lyon, France) was used to track total fish movement over light-dark cycles. The assay consisted of 2 min light and dark alternating periods, with a total of 3 light-dark cycles (Annex – Figure S1) after a 3 minute acclimation period. The light level was set for 50 % intensity, the integration time was set to 2 min, a transparent color background was used and the "detection threshold" value was set to 88.

In the protocol built on the ZebraLab□□v3 Automated Behavioral Analysis programme two monitoring zones were defined in the recording arena: an inner and an outer zone (see Annex- Figure S2) allowing the analysis of the tendency to swim near the edges of the container (as a measure of thigmotactic behavior). Behavioral endpoints measured included overall distance (mm) moved during a defined time period (swimming distance), and percentage of distance moved in the outer zone (calculated dividing the distance moved in the outer area by the overall swimming distance and multiplying by 100).

2.5.2. Exploratory Swimming Test

This test was based in the novel tank test (Wong et al., 2010) and aims at evaluating the exploratory behavior of fish when placed in a new environment. To conduct the test, a trapezoidal aquarium was used, presenting the following measures: 24.3 cm in the lower part, 27.2 cm in the upper part (in length). The diagonal side of the tank was 16 cm long and the opposite vertical side 17.2 cm high. The aquarium was placed on a flat, horizontal surface, and the camera (model Samsung Zoom Lens 4.9 24.5 mm 1:3.5 .9, 27 mm) was placed about 18.5 cm away, recording the lateral side of the aquarium.

Six fish of each concentration were individually placed in the trapezoidal aquarium and its behavior recorded for 5 minutes (Levin et al., 2007).

The videos obtained were analyzed using the ZebraLab®v3 Automated Behavioral Analysis programme. A protocol was created to analyze the movement of the fish (swimming time (secs)) in each of three distinct zones within the water column: zone 1 (bottom), zone 2 (middle) and zone 3 (top) as described in Domingues et al. (2016) (Annex – Figure S3). In this protocol, a transparent color background and a "detection threshold" value of 72 were used.

2.5.3. Feeding Test

A feeding assay was conducted based on the described in Domingues et al. (2016).Briefly, 6 adult zebrafish of each concentration were placed individually in an aquarium (length: 10.5 cm, width: 10.5 cm, height: 14.5 cm) with 7 cm water depth.

After an acclimation period of 3 min, 0.13 mg of food (GEMMA micro 500) were provided. Time until the first feeding action ($t_{initial}$) and time for total food ingestion (t_{final}) (until a maximum time of 20 minutes) were recorded (Domingues et al., 2016)(Chollett et al., 2014).

2.5.4. Shoaling Test

The tendency of the fish to approach a shoal of the same species (a measure of the social behavior of fish) was assessed by placing a zebrafish individually in an aquarium adjacent to another where a zebrafish shoal could be seen (Annex – Figure S4), as described by Calcagno et al. (2016). Individual fish were placed in a large aquarium (27 cm length, 13.3 cm width and 17.5 cm height) and the shoal was placed in a small aquarium (10.5 cm length, 10.5 cm width and 14.5 cm height), both with a water depth of 11 cm. Individual fish to be tested were placed in the middle of the aquarium and movement recorded for 10 min with a camera (model Samsung Zoom Lens 4.9 24.5mm 1:3.5 .9, 27mm) placed 32.5 cm away. Seven adults of each concentration were tested and

the time spent by each fish in three distinct zones of the aquarium was manually computed: zone 1- close proximity zone (the area closest to the shoal, 7 cm length); zone 2- neutral zone near the shoal (in the middle of the aquarium, 10 cm length) and zone 3- neutral zone away from the shoal (10 cm length) (Calcagno et al., 2016).

2.5.5. Predator Avoidance Test

In this test we intend to evaluate the reaction of adult zebrafish, in the presence of a model predator as described in Blaser (2006). A predator model was made with a 15 mL falcon tube, with plastic eyes and fins, painted in dark blue and filled with sand to increase its density (Annex – Figure S5). The predator model was placed in an aquarium (length: 27 cm, width: 13.3 cm, height: 17.5 cm, 11 cm water depth) adjacent to a larger aquarium (length: 27 cm, width: 13.3 cm, height: 17.5 cm, 11 cm water depth) where zebrafish were individually placed. Swimming of zebrafish was recorded for 10 min using a camera (model Samsung Zoom Lens 4.9 24.5mm 1:3.5 .9, 27mm) placed 30 cm away. Seven adults of each concentration were tested and the time spent by each fish in three distinct zones of the aquarium was manually computed: zone 1- close proximity zone (the area closest to the predator, 9 cm length); zone 2- neutral zone near the predator (in the middle of the aquarium, 9 cm length) and zone 3- neutral zone away from the predator (9 cm length) (Annex – Figure S6) (Sommer-Trembo et al., 2016).

2.6. Reproduction and F1 embryonic development

To assess if embryonic exposure to carbaryl had affected the quantity and quality of eggs and the embryo development of F1 generation adult fish (6 males and 6 females) were breed in aquariums with marbles in the bottom to prevent cannibalism as described in Andrade et al. (2017).The total number of eggs per concentration, the number of viable and non-viable eggs was registered.

To assess developmental effects in the F1 generation 20 fertilized eggs per concentration were placed in 24-well plates (one egg per well with 2 mL of culture water, Annex – Figure S7). The embryos and larvae were daily observed for 96 hours in the stereomicroscope (Microscope Stereo Zoom-SMZ 1500, Nikon), and the following parameters were recorded: somite formation, tail detachment, head , eyes and tail abnormalities, pericardial edema, heart rate (48 hpf) and coagulation/mortality (Annex - Table S4).

2.7. Statistical Analysis

SigmaPlot V.12.5 for Windows was used for statistical analyses. For the analysis of locomotor activity, a Two Way Repeat Measures (RM) ANOVA was used (after confirming normality and homoscedasticity of the data) to detect differences in total swimming distance (concentration vs stimulus as factors) and for the distance traveled in the outer zone, in dark periods (concentration and cycle as factors). After this, a multiple comparison test (Tukey test) was performed. To evaluate the thigmotactic behavior, we performed a One Way Repeated Measures (RM) ANOVA (stimulus as factor), followed by the Tukey test, to observe the differences between cycles.

Exploratory Swimming test, feeding test, shoaling test and predator avoidance test data were analyzed with One-Way ANOVA, followed by multiple-comparison test, in order to assess the differences in relation to the control group. To analyze the reproduction and F1 embryonic development, One-Way ANOVA was used. When there was no normality of data, Kruskal-Wallis One Way Analysis was performed.

The level of significance for all statistical analyzes was 0.05.

3. Results

3.1. Stability of carbaryl in the exposure medium

Chemical analysis of the exposure medium revealed that measured concentrations at the beginning of the test are among 51 and 67 % of the nominal concentrations while at the end of the test the measured concentrations drop to 7 to 10 % of the nominal concentrations (Annex – Table S3).

3.2. Locomotor activity and thigmotactic behavior

In the locomotor activity and thigmotactic behavior analyses, embryonic exposed fish were subjected to 3 cycles of light and dark periods to assess locomotor activity, thigmotaxis and habituation. Control organisms did not seem to react to periods of dark or light differently and their general locomotor activity seemed to decrease along the monitoring time (Fig. 5A). The 2-way repeated measures (RM) ANOVA indicated an interaction between carbaryl concentration and stimulus (dark/ light) ($p=0.02$) meaning that reaction to light/ dark depends on the concentration fish were pre-exposed to. In general fish did not respond differently to dark and light but in fish exposed to 1 mg/L a difference was verified with depressed activity being observed in light periods. Thus, effect of carbaryl exposure could not be detected in the dark periods but was detected in the light periods with depressed activities of fish exposed to 1.0 mg/L compared to control.

Locomotor analysis was done in two distinct zones (inner and outer zone) to account for the possibility of thigmotactic effects. Contrary to the observed in the distance traveled in the total area, the distance travelled in the outer zone was clearly different among periods of light and darkness (2-Way RM ANOVA, $p=0.018$) with all concentrations, including control, travelling longer distances in the dark (Fig. 5B).

In the dark periods and for the distance moved in the outer zone, the 2-way RM ANOVA indicated an interaction between carbaryl and cycle ($p<0001$). Indeed, a general

trend for decreasing travelling distances along the monitoring time can be observed (as also noticeable for the total distance); this trend however is more clearly observed in the control where differences among all cycles were verified.

The bar graph contains the same data previously presented in the graphs A and B but in such a way that is easier to observe the clear dose-dependent pattern of the distance travelled in the light periods but not in the dark (Fig. 5C). It is also noticeable the higher distance travelled by control fish in the outer zone during dark periods compared to light periods, meaning that although total distance in the control do not change from light to dark, fish swim more in the outer zone of the well than in the inner.

To evaluate thigmotactic behavior, distance travelled in the outer zone had to be analyzed in relation to the total area travelled, which may be (and is) different from treatment to treatment. Thus Fig. 6A presents the percentage of movement registered in the outer zone. In the control, a reaction to the dark stimulus is clearly observed, with a higher distance (in %) moved in the outer zone during dark periods (increased thigmotaxis). In the carbaryl treatments, differences in the thigmotaxis between light and dark were only observed for one cycle at the lowest concentrations and not detectable at all at the highest concentration.

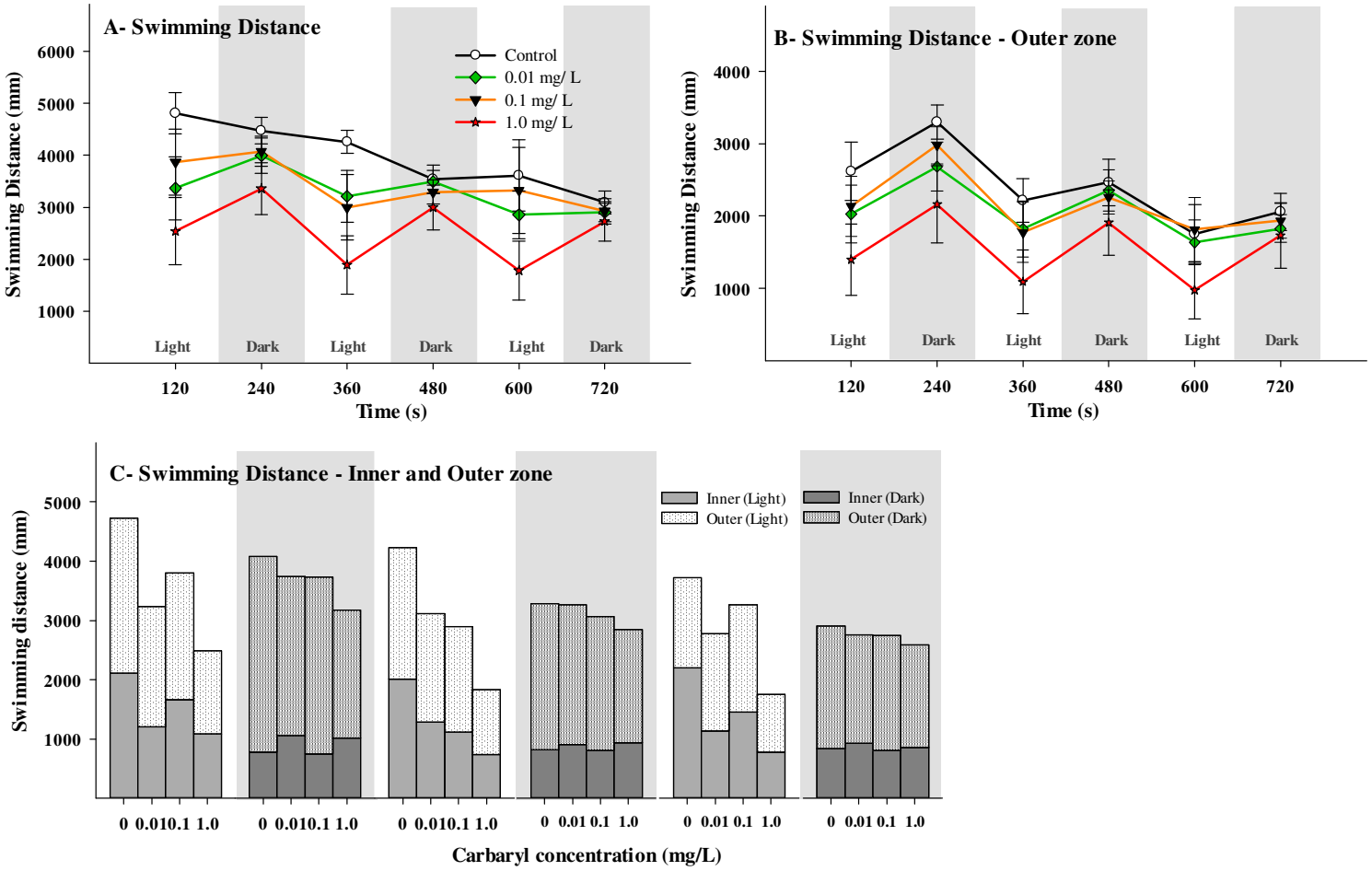

Figure 5 – Effects of embryonic exposure to sublethal concentrations of carbaryl on response to light and dark cycles in adult zebrafish. **A –** Total swimming distance traveled by the adult zebrafish; **B -** Analysis of the thigmotactic response, with the distance traveled in the outer zone; **C –** Distance traveled in the two different areas (inner and outer zone) (this bar graph contains the same data as before (A and B), allowing more simplified analysis). The presented values are mean values ± standard error (SE).

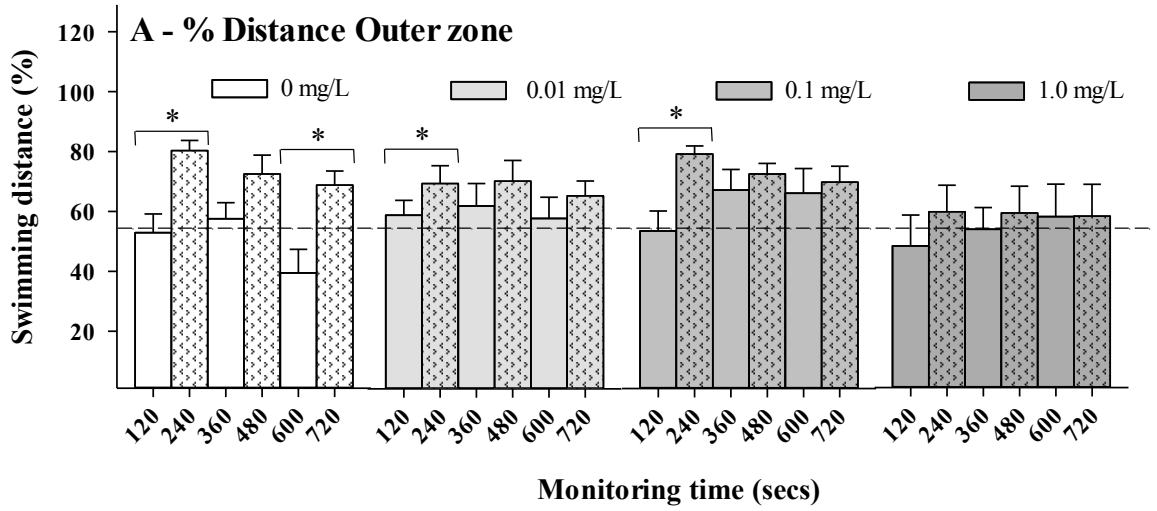

Figure 6 – Percentage of swimming distance by zebrafish pre-exposed to sublethal concentrations of carbaryl. **A –** Percentage of distance traveled in the outer zone, during the dark and light periods (mean value ± standard error) (Tukey Test: *p < 0.05).

3.3. Exploratory Swimming Test

Embryonic exposure to sublethal concentrations of carbaryl showed a trend on the behavior of adult zebrafish when placed in a new environment. The control fish spent approximately similar time periods in the 3 zones whereas fish exposed in the embryonic stage seem to spend less time in the top layer of the aquarium, as can be seen in Fig.7A. However, there are no significant differences in the time spent in each layer, between the concentrations tested (One Way ANOVA, Bottom: p=0.924; Middle: p=0.066; Top: p=0.052).

3.4. Feeding Test

The effects of sublethal concentrations of carbaryl on feeding behavior in adult zebrafish are show in Fig. 7B. Time for the first feeding action and total food ingestion seems to slightly increase in fish exposed to carbaryl at the embryo stage. This trend

follows a dose-dependent concentration, however no statistical differences were observed (One Way ANOVA, p= 0,476 for $t_{initial}$ and One Way ANOVA, p= 0,346 for t_{final}).

3.5. Shoaling Test

Fish exposed to the highest concentration seem to spend more time in the area near the shoal (Zone 1), compared with the control group, where fish tend to swim equally the 2 neutral areas of the aquarium (Neutral zone near the shoal (Zone 2) and Neutral zone away from the shoal (Zone 3), as can be seen in Fig. 7C. However, there are no significant differences in the time spent in each zone, between the concentrations tested (One Way ANOVA, Zone 1: p=0.116; Zone 2: p=0.169; Zone 3: p=0.061).

3.6. Predator Avoidance Test

According to Fig. 7D, in the highest concentration (1.0 mg/L), it can be observed that the fish have a trend to spend a longer time in the area that was farthest from the predator (Zone 3 – Neutral zone away from the predator). However the data obtained do not allow us to determine whether pre-exposure affected predator avoidance and there are no significant differences in the time spent in each zone, between the concentrations tested (One-Way ANOVA, Zone 1: p=0.422; Zone 2: p=0.263; Zone 3: p=0.953).

3.7. Reproduction and F1 embryonic development

The results obtained in offspring (F1 generation) of the zebrafish pre-exposed to carbaryl showed a tendency to decrease the heart rate of the larvae. However no

statistically significant differences were observed (One-Way ANOVA: Total number of eggs: p=0.295; % Viability: p=0.381; % Abnormalities: p=0.889; Heart rate: p=0.407; % Mortality: p=0.698) (Annex – Table S4).

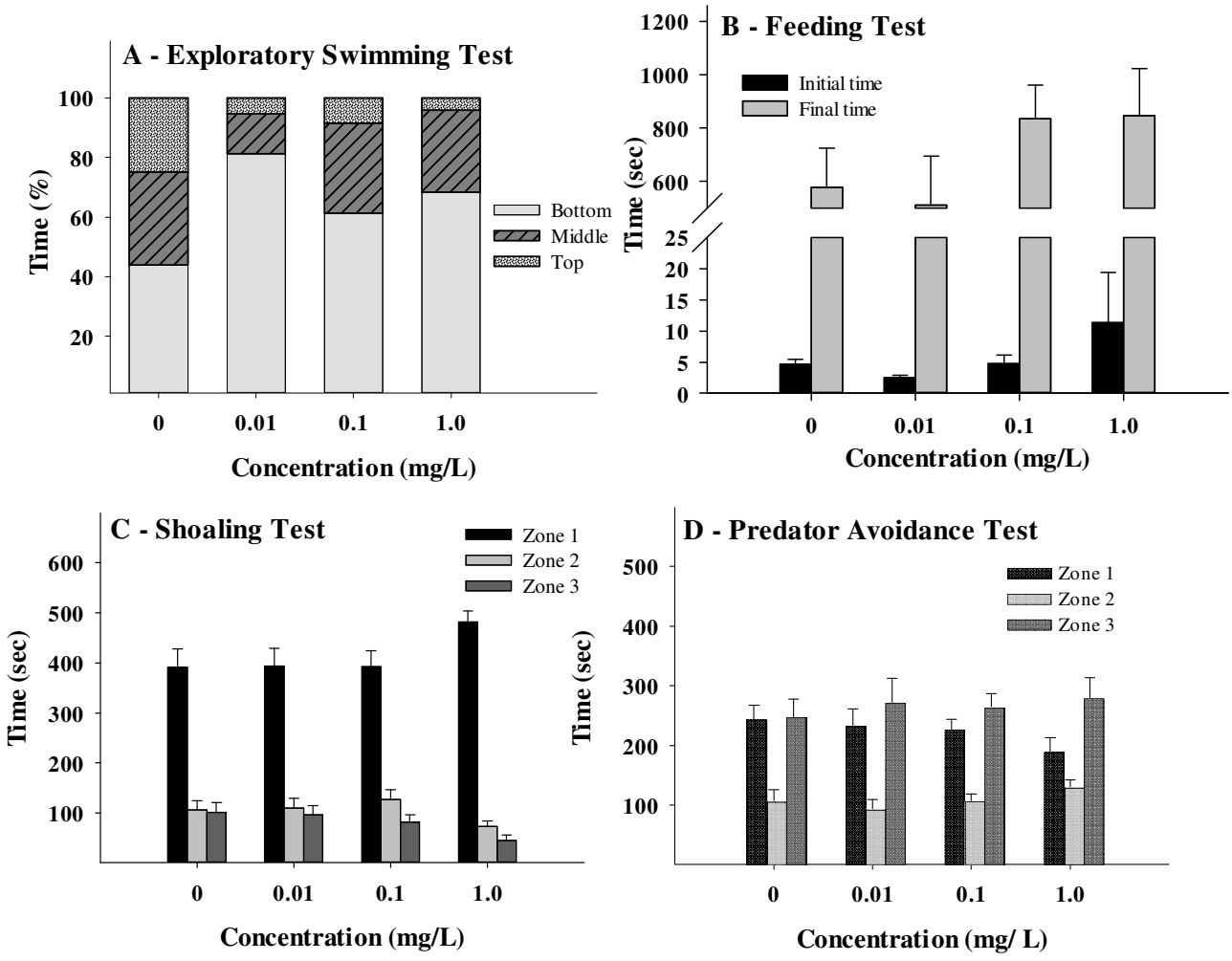

Figure 7 – Effects of embryonic exposure to sublethal concentrations of carbaryl on adult zebrafish behavior. **A – Exploratory Swimming Test:** swimming behavior of adult zebrafish, with the three layers (bottom, middle and top) (mean value ± standard error (SE)); **B – Feeding Test:** feeding behavior of adult zebrafish. The results show the time spent on the first feeding action (black bars) and on food intake (grey bars), expressed as mean value ± SE; **C – Shoaling Test:** social behavior of adult zebrafish discriminated between the zone 1 – Close Proximity Zone, zone 2 – Neutral zone near the shoal and zone 3 – Neutral zone away from the shoal (mean value ± SE); **D - Predator Avoidance Test:** reaction of adult zebrafish to the presence of a predator, discriminated in Zone 1 – Close proximity zone (the area closest to the predator); Zone 2 - Neutral zone near the predator (in the middle of the aquarium) and Zone 3 – Neutral zone away from the predator (mean value ± SE).

4. Discussion

In this study a battery of tests to assess social, anti-predatory, feeding, anxiety-like behavior and locomotion and thigmotaxis was used in adult zebrafish to evaluate the effects of a developmental exposure to the pesticide carbaryl. Behavior is considered a very sensitive endpoint and has been increasingly used to evaluate subtle sublethal effects of environmental contaminants. To our knowledge, although some studies reporting the behavioral effects of carbamates can be found, none was devoted to the assessment of a developmental exposure in adult fish.

Locomotion evaluation under dark/light cycles aimed to assess: *1)* effects of embryonic exposure to carbaryl on the distance moved; *2)* effects in the thigmotactic behavior and *3)* existence of habituation to stimuli. Since zebrafish is a diurnal animal, it tends to avoid darkness and have a daytime preference which would allow it to obtain food, avoid possible predators and find shoals of the same species (Gerlai et al., 2000). Thus, similarly to what happens with zebrafish larvae we would expect control zebrafish to have higher locomotion activity under darkness as a response to a stress stimulus. However, responses of control organisms to light/dark changes were only observed when analyzing thigmotactic behavior (% of distance moved in the outer zone of the arena) and not when looking at the general locomotion (total distance travelled) Thigmotactic behavior (or thigmotaxis) is a tendency of an animal, when placed in a new environment, remain close to the middle walls, avoiding the center of the aquarium. (Best et al., 2017; Champagne et al., 2010) and is, in fish, considered an anxiety-like behaviors (Best et al., 2017; Blaser, 2006; Félix, 2016). Thus, our data suggest that the light/dark stimulus constitutes a stress event for adult zebrafish because it induced thigmotaxis although not influencing the total distance covered. Moreover, adaptation to the stressful event was observed along the monitoring time expressed by a decrease in the magnitude of the thigmotactic response and supporting memory and learning ability of zebrafish

Effects of carbaryl embryonic exposure were evident at the highest concentration with lower distances moved in the light periods, lower habituation to the stimulus along the monitoring time and lower thigmotactic response (compared to the previous light period).

The battery of behavioral tests performed in this work included the exploratory (novel environment), the shoal and the predator model tests to assess respectively anxiety-

like, social and antipredatory responses. Moreover a feeding test was realized. In all these tests no significant effects were obtained despite the clear dose-response trends observed. Given the consistency of these trends one may assume that significances were not observed probably due to high variability of data or low N used and that a discussion of these trends obtained is worthwhile. In the exploratory swimming test, a trend in swimming behavior was observed in all concentrations tested (0.01, 0.1 and 1.0 mg/L), with a higher percentage of time spent in the bottom layer and lower in the top layer. Effects of carbamates on locomotion parameters have been previously reported in fish although in studies using juvenile or adult exposures and not developmental exposures as in the present work. For instance Labenia et al. (2007) exposed adult cutthroat trout (*Oncorhynchus clarki clarki*) to carbaryl (500, 750 and 100 µg/L), for 6 hours and found that, at the two highest concentrations, the fish exhibited a decrease in swimming activity and of their capacity for guidance. A decrease in swim speed was observed in juvenile sea bass (*Dicentrarchus labrax* L.) exposed for 96 hours to carbofuran (31, 63, 125 and 250 µg/L) (Hernández-Moren et al., 2011) while in a study with juveniles goldfish (*Carassius auratus*), also exposed to carbofuran (1, 10 and 100 µg/L) for 4, 8 and 12 hours of exposure, a change in swimming behavior was more evident in the longer exposure time (12 hours) and at the highest concentrations, with an increase in the burst swimming phenomenon (sudden change of non-directed movement), compared to control (Saglio and Trijasse, 1996).

The observed disruption in the swimming behavior of zebrafish may be related to the alterations in the feeding behavior reported: at 1.0 mg/L the time for the first feeding action and the time for total food intake were higher compared to the control. Effects in feeding have also been reported in other studies: juvenile rainbow trout (*Oncorhynchus mykiss*) exposed for 96 hours to 1.0 mg/L of carbaryl reduced the percentage of daphnia consumed (Little et al., 1990) while juveniles of goldfish (*Carassius auratus*) exposed for 4, 8 and 12 hours to carbofuran (1, 10 and 100 µg/L) showed a decreased interest by the food provided (filtrate of chironomids) (Saglio and Trijasse, 1996). Feeding disruption is a high relevant ecological indicator that can be directly linked to impacts at predator-prey level. In the wild, organisms affected will have a greater difficulty in catching live prey with a consequent decrease in competitive advantage and an increase in susceptibility to

predation, affecting the entire aquatic food chain (Domingues, Oliveira, Soares, & Amorim, 2016).

These results show that a developmental exposure to concentrations of carbaryl (which do not produce any observable effect on embryos and larvae) has indeed a long lasting effect on adults. The mechanism behind these effects is difficult to establish. Carbaryl is known to act through the inhibition of acetylcholinesterase activity in the brain and in the muscles, leading to an accumulation of the neurotransmitter acetylcholine (Ach) in the synaptic cleft, with continuous stimulation of neurons occurring, which damages the neuromuscular function (Hernández-Moreno et al., 2011). As a consequence of this effect, locomotion is strongly affected, reducing the ability to escape from predators and obtaining food, leading to an increase in population mortality (Hernández-Moreno et al., 2011). However this inhibition is reversible in carbamates and long lasting effects of this inhibition at developmental stage are unknown. Alternatively, exposed embryos may have suffered damage at electrophysiological level such as in Mauthner cells, located in the fish hindbrain. These cells receive information from the receptors for audition, vision, gravity, vibration, angular acceleration, water flow and electric fields. When a stimulation of Mauthner cells occurs, it generates an excitation of the primary and secondary motoneurons and the interneurons that are connected to the muscles. Carbaryl may affect the transmission of nerve impulses from the Mauthner cells to the muscles, causing a neuromuscular delay, affecting locomotion (Carlson et al., 1998).

In the present study, a tendency to increase social behavior (measured through the avoidance or attraction to a shoal of the same species) was observed in fish with a developmental exposure to 1.0 mg/L of carbaryl which had a longer permanence in the area that was near the shoal, in relation to the other concentrations tested. Bretaud et al. (2002) carried out a study with carbamate carbofuran, obtaining similar results. They exposed goldfish (*Carassius auratus*) at 3 concentrations of carbofuran (5, 50 and 500 µg/L) for 24 and 48 hours and observed a significant increase in the shoaling/ grouping behavior at both exposure times, in the highest concentration (500 µg/L). These effects observed in the goldfish social behavior were related to catecholamine levels (norepinephrine (NE) and dopamine) in the brain of exposed fish. An increase in NE levels at concentrations of 50 and 500 µg/L was observed after 24 hours of exposure and a significant increase in dopamine levels at the highest concentrations, after 48 hours of

exposure. The change of these levels may be related to the increase of AChE, due to exposure to carbofuran (Bretaud et al., 2002). This correlation was the object of a study carried out by El-Etri et al. (1999). They applied an injection of an AChE inhibitor on the olfactory bulb of rats and verified a significant increase in the extracellular basal levels of NE, thus concluding that AChE regulates NE release.

Previous works with carbaryl account to the influence of carbamates in the antipredatory response of fish. Eg, juvenile rainbow trout (*Oncorhynchus mykiss*), exposed for 96 hours to carbaryl (0.01, 0.1 and 1.0 mg/L) showed an increased vulnerability to the predator used in the test (*Micropterus salmoides*) at all concentrations tested (Little et al., 1990) while adult cutthroat trout (*Oncorhynchus clarki clarki*) exposed to 200, 500 and 1000 µg/L for 2 hours showed a dose-response in avoidance (less avoidance with increasing concentrations) and were more predated by the introduced predator, the lingcod (*Ophiodon elongatus*) (Labenia et al., 2007). Fish, when attacked by predators, release "alarm substances", released by the epidermal cells, allowing the escape of the remaining individuals. When disruption occurs in the detection and response of this chemical signal, susceptibility to predation increases. In addition, exposure to carbaryl may also have affected visual and sensory perception (Scott and Sloman, 2004). This type of behavioral response has an important ecological relevance and can affect higher trophic levels such as piscivorous birds, marine mammals and even humans (Labenia et al., 2007).

Regarding the reproductive output, zebrafish exposed as embryos to carbaryl were bred to assess effects in the number and viability of eggs and also in the development of F1 embryos. No clear effects were observed, probably because at exposing time organisms did not present a complete differentiation and formation of the reproductive system. Effects of carbamates in adult fish reproduction are, however, well documented (Adhikari et al., 2008; Kaur and Dhawan, 1996).

The behavior is an integration of several biochemical, physiological and morphological processes, and thus, behavioral observations provide a connection with many cellular processes, essential to the viability of the organism, the population and community (Little, 1990).

Sensory, neurological, hormonal/ endocrine and metabolic systems are examples of physiological systems that are implicated in behavior and, when suffering a dysfunction by environmental contaminants, may lead to severe damage in the survival of organisms

(Scott and Sloman, 2004). At the sensory level, olfactory is a basic sense that allows fish to recognize a new environment, escape from predators, reproduction and dominance in the environment. Fish, when suffering sublethal exposure, can suffer damage in the olfactory cells and cell death, impairing the normal functioning of the olfactory system. This possible accumulation of the contaminant in olfactory cells and the consequences that may occur, may cause a failure in the transmission of sensory information from the olfactory epithelium to the nervous system (Scott and Sloman, 2004).

Neurological dysfunction following exposure to a contaminant can lead to behavioral changes, since neurotransmitters levels and enzyme function play an important role in behavior. Inhibition induced by carbamates is transient, with partial recovery of neurotransmitters levels occurring. However, effects at developmental level need to be elucidated as lasting neurotoxic and neurobehavioral effects are be produced affecting other important pathways and biochemical and molecular mechanisms (Abreu-Villaça and Levin, 2017; Labenia et al., 2007).

Modification of other neurotransmitters catecholamines (NE and dopamine) may also happen as demonstrated in the studies performed by Bretaud et al. (2002) and El-Etri et al. (1999), mentioned above. In the same way, Gopal et al. (1995) observed a generalized decrease in the levels of serotonin, dopamine and norepinephrine in the cortex region of the brain of *Channa punctatus* after exposure to carbofuran. This neurotoxic effect affected brain regions responsive to regulation of motor activity and behavior.

Endocrine disruption may also be manifested at the behavioral level in organisms. The endocrine system integrates a large set of organs and glands, regulating the body's balance (e.g. temperature, glucose, cellular metabolism, reproduction and others), allowing its normal functioning. The maintenance and regulation of homeostasis is performed in conjunction with the nervous system, pituitary hormones and also hormones produced by other glands, allowing the communication and response to the environment where the organism are located (Shahidehnia, 2016). As an endocrine disrupting chemical (EDC) (Shahidehnia, 2016) carbaryl may have produced the observed effects in this work by interfering with the embryos homeostasis with last longing effects. In fact, there are pathways that can be affected, such as changes in growth hormone and thyroid hormone levels (thyroxine and triiodothyronine), which play important roles in fish behavior. The blockage of these pathways may result in behavioral toxicity.

Metabolism can also be affected by chemical stress by disrupting several functions, from the tissue level (metabolic substrates and enzymatic activity) to the global body's response (metabolic rate and behavior). Disruption in the normal functioning of metabolic processes (e.g. supply and energy availability) can strongly influence fish behavior (Scott and Sloman, 2004). There are studies that demonstrate the action of carbamates in altering levels of metabolic substrate, compromising their storage and mobilization. In addition, they may induce toxic effects on enzyme activity. As described in Singh et al. (1998), sublethal concentrations of carbofuran caused a significant reduction in protein content and an inhibition of lactate dehydrogenase activity in the various tissues of *Clarias batrachus*. Also in a study performed with the species mentioned above, Jyothi et al. (1999) showed a significant increase in glycoside levels, serum alkaline phosphatase level and total bilirubin level after carbaryl exposure.

Sometimes exposure to chemical compounds, in addition to affecting the body's physiological systems, can also cause damage to the DNA of the cells. If no repair of these damages occurs, a cascade of biological consequences begins in the cells, organs, organism, eventually reaching higher trophic levels such as population and community (Lee and Steinert, 2003). Carbamates are a class of pesticides than can produce these lesions. According to Nwani et al. (2014), juveniles *Channa punctatus* presented severe nuclear lesions and significantly increased micronucleus induction, with chromosomal damage, in erythrocytes after 96h exposure to carbofuran. Similar effects were also observed in juveniles common carp (*Cyprinus carpio*), after a 96h exposure to propoxur (Gül et al., 2012).

5. Conclusion

Developmental exposure of zebrafish to sublethal concentrations of carbaryl produced a behavioral disruption in the adulthood. Main effects included decreased locomotion and decreased thigmotaxis during light/dark transitions. Other trends, although not statistically significant were considered important due to their consistency. This is the case of social behavior that seemed to be increased and exploratory and feeding behaviors

that seem to be decreased. Thigmotaxis and exploratory behavior are considered markers for anxiety in fish. This work clearly demonstrates the effects of a (chemical) stress event at embryonic stages in the development programming of fish and their long last effects. In a context of ecological risk assessment, this work shows that relevant scenarios including long term effects measured with sensitive and informative parameters such as behavior have to be considered for an accurate risk evaluation Further work should focus on the mechanistic effects of developmental disruption that explain the long term effects observed.

Acknowledgments

This study was supported by a PhD grant (PD/BD/127811/2016) attributed to Ana Rita Marques Almeida and by the Pos-Doc grant (SFRH/BPD/90521/2012) attributed to Inês Domingues by the Portuguese Science and Technology Foundation (FCT), funding by FEDER through COMPETE e Programa Operacional Factores de Competitividade and by National funding through FCT, within the research project Climatox—Impact of climatic changes on toxicity of pollutants (Ref. FCT PTDC/AAG-GLO/4059/2012).

Support was also given by the Ministry of Education, Youth and Sports of the Czech Republic - projects "CENAKVA"(No. CZ.1.05/ 2.1.00/01.0024) and "CENAKVA II" (No. LO1205 under the NPU I program).

Statement of ethic on animal use

All procedures respect the European Directive on the protection of animals used for scientific purposes (2010/63/EU) and its transposition to the Portuguese law (Decreto-lei 113/2013), ensuring minimal animal stress and discomfort.

References

Abreu-Villaça, Y., Levin, E.D., 2017. Developmental neurotoxicity of succeeding generations of insecticides. Environ. Int. 99, 55–77. https://doi.org/10.1016/j.envint.2016.11.019

Adhikari, S., Sarkar, B., Chattopadhyay, A., Chattopadhyay, D.N., Sarkar, S.K., Ayyappan, S., 2008. Carbofuran induced changes in breeding of a freshwater fish, Labeo rohita (Hamilton). Toxicol. Environ. Chem. 90, 457–465. https://doi.org/10.1080/02772240701589487

Andrade, T. de S., 2015. EFFECTS OF ENVIRONMENTAL FACTORS ON THE TOXICITY OF PESTICIDES TO ZEBRAFISH EMBRYOS. University of Aveiro.

Andrade, T.S., Henriques, J.F., Almeida, R., Machado, A.L., Giang, P.T., Soares, A.M.V.M., Domingues, I., 2015. Carbendazim exposure induces developmental , biochemical and behaviour disturbance in zebrafish embryos. Elsevier B.V. 122–157. https://doi.org/10.1016/j.aquatox.2015.11.017

Andrade, T.S., Henriques, J.F., Almeida, R., Scholz, S., Amadeu, M.V.M., 2017. Zebrafish embryo tolerance to environmental stress factors – concentration / dose response analysis of oxygen limitation , pH and UV- light irradiation 60–91.

Bailey, J.M., Oliveri, A.N., Zhang, C., Frazier, J.M., Mackinnon, S., Cole, G.J., Levin, E.D., 2015. Long-term behavioral impairment following acute embryonic ethanol exposure in zebrafish. Neurotoxicol. Teratol. 48, 1–8. https://doi.org/10.1016/j.ntt.2015.01.005

Best, C., Kurrasch, D.M., Vijayan, M.M., 2017. Maternal cortisol stimulates neurogenesis and affects larval behaviour in zebrafish. Sci. Rep. 7, 40905. https://doi.org/10.1038/srep40905

Blaser, R., 2006. Behavioral phenotyping in zebrafish : Comparison of three behavioral quantification methods. Behav. Res. Methods 38, 456–469.

Bouchard, M., Carrier, G., Brunet, R.C., 2008. Assessment of absorbed doses of carbaryl and associated health risks in a group of horticultural greenhouse workers. Int. Arch. Occup. Environ. Health 81, 355–370. https://doi.org/10.1007/s00420-007-0220-1

Branch, R.A., Jacqz, E., 1986. Is carbaryl as safe as its reputation? Does it have a potential for causing chronic neurotoxicity in humans? Am. J. Med. 80, 659–664. https://doi.org/https://doi.org/10.1016/0002-9343(86)90821-1

Bretaud, S., Saglio, P., Saligaut, C., Auperin, B., 2002. Biochemical and behavioral effects of carbofuran in goldfish (Carassius auratus). Environ. Toxicol. Chem. 21, 175–181. https://doi.org/10.1002/etc.5620210125

Çakıcı, Ö., 2016. Histopathological study of toxic effects of carbaryl on digestive tract of Bufotes variabilis (Anura: Bufonidae). Environ. Sci. Pollut. Res. 23, 13432–13437. https://doi.org/10.1007/s11356-016-6529-0

Calcagno, E., Durando, P., Valdés, M.E., Franchioni, L., Bistoni, M. de los Á., 2016. Effects of carbamazepine on cortisol levels and behavioral responses to stress in the fish Jenynsia multidentata. Physiol. Behav. 158, 68–75. https://doi.org/10.1016/j.physbeh.2016.02.025

Carlson, R.W., Bradbury, S.P., Drummond, R.A., Hammermeister, D.E., 1998. Neurological effects on startle response and escape from predation by medaka exposed to organic chemicals. Aquat. Toxicol. 43, 51–68. https://doi.org/10.1016/S0166-445X(97)00097-0

Champagne, D.L., Hoefnagels, C.C.M., de Kloet, R.E., Richardson, M.K., 2010. Translating rodent behavioral repertoire to zebrafish (Danio rerio): Relevance for stress research. Behav. Brain Res. 214, 332–342. https://doi.org/http://doi.org/10.1016/j.bbr.2010.06.001

Chollett, D., Perez, K.E., King-Heiden, T.C., 2014. Embryonic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin impairs prey capture by zebrafish larvae. Environ. Toxicol. Chem. 33, 784–790. https://doi.org/10.1002/etc.2477

De Jesús Andino, F., Lawrence, B.P., Robert, J., 2017. Long term effects of carbaryl exposure on antiviral immune responses in Xenopus laevis. Chemosphere 170, 169–175. https://doi.org/10.1016/j.chemosphere.2016.12.018

Domingues, I., Oliveira, R., Soares, A.M.V.M., Amorim, M.J.B., 2016. Effects of ivermectin on Danio rerio: a multiple endpoint approach: behaviour, weight and subcellular markers. Ecotoxicology 25, 491–499. https://doi.org/10.1007/s10646-015-1607-5

El-Etri, M.., Ennis, M., Griff, E.., Shipley, M.., 1999. Evidence for cholinergic regulation of basal norepinephrine release in the rat olfactory bulb. Neuroscience 93, 611–617. https://doi.org/10.1016/S0306-4522(99)00169-4

Félix, L.M.L., 2016. Towards an understanding of ketamine teratogenicity in zebrafish early development. UNIVERSITY OF TRÁS-OS-MONTES AND ALTO DOURO.

Fleeger, J.W., Carman, K.R., Nisbet, R.M., 2003. Indirect effects of contaminants in aquatic ecosystems. Sci. Total Environ. 317, 207–233. https://doi.org/10.1016/S0048-9697(03)00141-4

Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. Pharmacol. Biochem. Behav. 67, 773–782. https://doi.org/10.1016/S0091-3057(00)00422-6

Gopal, K., Ram, M., 1995. Alteration in the neurotransmitter levels in the brain of the freshwater snakehead fish (Channa punctatus) exposed to carbofuran. Ecotoxicology 4, 1–4. https://doi.org/10.1007/BF00350646

Gül, A., Benli, A.Ç.K., Ayhan, A., Memmi, B.K., Selvi, M., Sepici-Dinçel, A., Çakiroğullari, G.Ç., Erkoç, F., 2012. Sublethal propoxur toxicity to juvenile common carp (Cyprinus carpio L., 1758): Biochemical, hematological, histopathological, and genotoxicity effects, Environmental Toxicology and Chemistry.

https://doi.org/10.1002/etc.1924

Hernández-Moreno, D., Pérez-López, M., Soler, F., Gravato, C., Guilhermino, L., 2011. Effects of carbofuran on the sea bass (Dicentrarchus labrax L.): Study of biomarkers and behaviour alterations. Ecotoxicol. Environ. Saf. 74, 1905–1912. https://doi.org/10.1016/j.ecoenv.2011.07.016

Jeon, J., Kretschmann, A., Escher, B.I., Hollender, J., 2013. Characterization of acetylcholinesterase inhibition and energy allocation in Daphnia magna exposed to carbaryl. Ecotoxicol. Environ. Saf. 98, 28–35. https://doi.org/10.1016/j.ecoenv.2013.09.033

Jyothi, B., Narayan, G., 1999. Certain pesticide-induced carbohydrate metabolic disorders in the serum of freshwater fish Clarias batrachus (Linn.). Food Chem. Toxicol. 37, 417–421. https://doi.org/10.1016/S0278-6915(99)00020-4

Kaur, K., Dhawan, A., 1996. Effect of Carbaryl on Tissue Composition, Maturation, and Breeding Potential of Cirrhina mrigala (Ham.). Bull. Environ. Contam. Toxicol. 57, 480–486. https://doi.org/10.1007/s001289900215

Klüver, N., König, M., Ortmann, J., Massei, R., Paschke, A., Kühne, R., Scholz, S., 2015. Fish Embryo Toxicity Test: Identification of Compounds with Weak Toxicity and Analysis of Behavioral Effects To Improve Prediction of Acute Toxicity for Neurotoxic Compounds. Environ. Sci. Technol. 49, 7002–7011. https://doi.org/10.1021/acs.est.5b01910

Labenia, J., Baldwin, D., French, B., Davis, J., Scholz, N., 2007. Behavioral impairment and increased predation mortality in cutthroat trout exposed to carbaryl. Mar. Ecol. Prog. Ser. 329, 1–11. https://doi.org/10.3354/meps329001

Lee, R.F., Steinert, S., 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat. Res. Mutat. Res. 544, 43–64. https://doi.org/10.1016/S1383-5742(03)00017-6

Levin, E.D., Bencan, Z., Cerutti, D.T., 2007. Anxiolytic effects of nicotine in zebrafish. Physiol. Behav. 90, 54–58. https://doi.org/10.1016/j.physbeh.2006.08.026

Little, E.E., 1990. BEHAVIORAL TOXICOLOGY: STIMULATING CHALLENGES FOR A GROWING DISCIPLINE. Environ. Toxicol. Chem. 9, 1–2.

Little, E.E., Archeski, R.D., Flerov, B.A., Kozlovskaya, V.I., 1990. Behavioral indicators of sublethal toxicity in rainbow trout. Arch. Environ. Contam. Toxicol. 19, 380–385. https://doi.org/10.1007/BF01054982

Mahajan, R., Blair, A., Coble, J., Lynch, C.F., Hoppin, J.A., Sandler, D.P., Alavanja, M.C.R., 2007. Carbaryl exposure and incident cancer in the Agricultural Health Study. Int. J. Cancer 121, 1799–1805. https://doi.org/10.1002/ijc.22836

Maximino, C., de Brito, T.M., da Silva Batista, A.W., Herculano, A.M., Morato, S., Gouveia, A., 2010. Measuring anxiety in zebrafish: A critical review. Behav. Brain Res. 214, 157–171. https://doi.org/10.1016/j.bbr.2010.05.031

Muthaiah, V.P.K., Ding, D., Salvi, R., Roth, J.A., 2016. Carbaryl-induced Ototoxicity in Rat Postnatal Cochlear Organotypic Cultures. Environ. Toxicol. 24, 296–303. https://doi.org/10.1002/tox.22296

Nwani, C.D., Nagpure, N.S., Kumar, R., Kushwaha, B., Kumar, P., Lakra, W.S., 2014. Induction of micronuclei and nuclear lesions in Channa punctatus following exposure to carbosulfan, glyphosate and atrazine. Drug Chem. Toxicol. 37, 370–377. https://doi.org/10.3109/01480545.2013.866138

OECD, 2013. Oecd guidelines for the testing of chemicals.

Powers, C.M., Levin, E.D., Seidler, F.J., Slotkin, T.A., 2011. Silver exposure in developing zebrafish produces persistent synaptic and behavioral changes. Neurotoxicol. Teratol. 33, 329–332. https://doi.org/10.1016/j.ntt.2010.10.006

Saglio, P., Trijasse, S., 1996. Behavioral Effects of Waterborne Carbofuran in Goldfish. Arch. Environ. Contam. Toxicol. 31, 232–238. https://doi.org/10.1007/s002449900103

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68, 369–392. https://doi.org/10.1016/j.aquatox.2004.03.016

Shahidehnia, M., 2016. Epigenetic Effects of Endocrine Disrupting Chemicals. J. Environ. Anal. Toxicol. https://doi.org/10.4172/2161-0525.1000381

Singh, R.K., Sharma, B., 1998. Carbofuran-induced biochemical changes in Clarias batrachus. Pestic. Sci. 53, 285–290. https://doi.org/10.1002/(SICI)1096-9063(199808)53:4<285::AID-PS771>3.0.CO;2-0

Sommer-Trembo, C., Zimmer, C., Jourdan, J., Bierbach, D., Plath, M., 2016. Predator experience homogenizes consistent individual differences in predator avoidance 34, 155–165. https://doi.org/10.1007/s10164-016-0460-1

Steenbergen, P.J., Richardson, M.K., Champagne, D.L., 2011. The use of the zebrafish model in stress research. Prog. Neuropsychopharmacol. Biol. Psychiatry 35, 1432–1451. https://doi.org/10.1016/j.pnpbp.2010.10.010

Toumi, H., Bejaoui, M., Touaylia, S., Burga Perez, K.F., Ferard, J.F., 2016. Effect of carbaryl (carbamate insecticide) on acetylcholinesterase activity of two strains of Daphnia magna (Crustacea, Cladocera). J. Environ. Sci. Heal. Part B 51, 777–780. https://doi.org/10.1080/03601234.2016.1198645

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K.M., Stewart, A., Gilder, T., Beeson, E., Zapolsky, I., Wu, N., Cachat, J., Kalueff, A. V, 2010. Analyzing habituation responses to novelty in zebrafish (Danio rerio), Behavioural Brain Research. Elsevier B.V. https://doi.org/10.1016/j.bbr.2009.12.023

Annex

Behavioral effects in adult zebrafish after developmental exposure to carbaryl

(To be submitted to *NeuroToxicology*)

Daniela Correia[1], Ana Rita Almeida[1], Joana Santos[1], Vladimir Zlabek[2], Marcelino Miguel Oliveira[1] and Inês Domingues[1]

1 Department of Biology & CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

2 University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, Vodnany 389 25, Czech Republic

Table S1 – Chromatographic gradient for the elution of target compounds.

Time (min)	Mobile phase composition		Flow rate, (µL/min)
	Water (0.1 % FA*)	Acetonitrile (0.1 % FA)	
0.00	95	5	300
1.00	95	5	300
4.00	20	80	400
7.00	0	100	400
7.01	95	5	300
9.00	95	5	300

* formic acid

Table S2 – LC-MS method parameters of targeted compounds.

Compound	Mode	Quantification transition	Confirmation transition	RF Lens (V)	Retention time
Carbaryl	+	202.13→145.111	202.13→127.111	30	3.94
Diclofenac ($^{13}C_6$)	+	302.07→220.058	*	50	4.41

Table S3 – Analytical measurement of exposure mediums of the Fish Embryo Toxicity Test (Acute assay) to carbaryl.

Nominal Exposure Concentrations (mg/L)	Measured Concentrations (mg/L)		% of Nominal concentrations	
	0h	24 h	0h	24h
0.01	0.006	0.001	60	10
0.1	0.051	0.009	51	9
1	0.671	0.076	67.1	7.6

Table S4 – Effects of sublethal concentrations on F1 generation and development endpoints (mean ± standard error).

Concentration (mg/L)	Eggs total number	% Viability	% Abnormalities	Heart rate	% Mortality
0	52.8 ± 22.417	68.6 ± 17.604	5 ± 5	148 ± 5.769	25 ± 25
0.01	151.8 ± 38.473	87.9 ± 4.494	4 ±2.449	145.3 ± 3.125	14 ± 5.099
0.1	293.4 ±141.56	74.1 ± 18.605	6.25 ± 3.146	143.2 ± 3.233	5 ± 2.041
1.0	183.8 ± 74.4	77.7 ± 8.710	6 ± 2.915	139.5 ± 2.537	7 ± 3.742

Behavior Tests

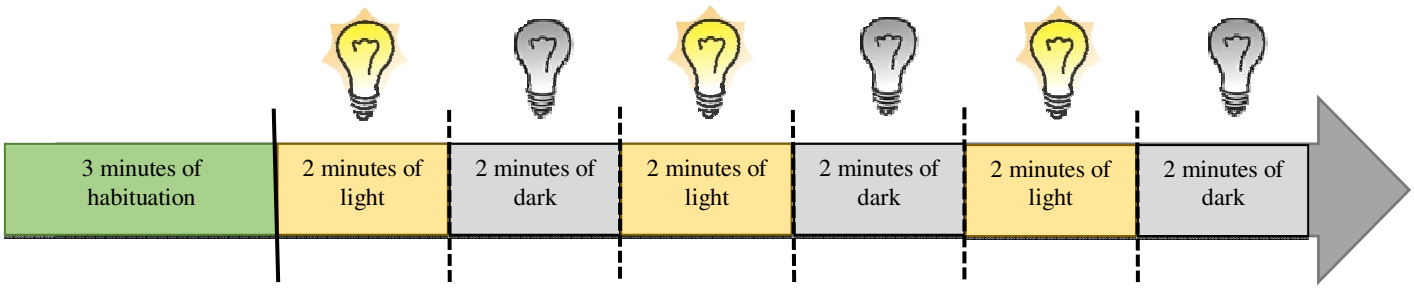

Figure S1 – Illustrative diagram of the execution of locomotor activity and thigmotactic behavior.

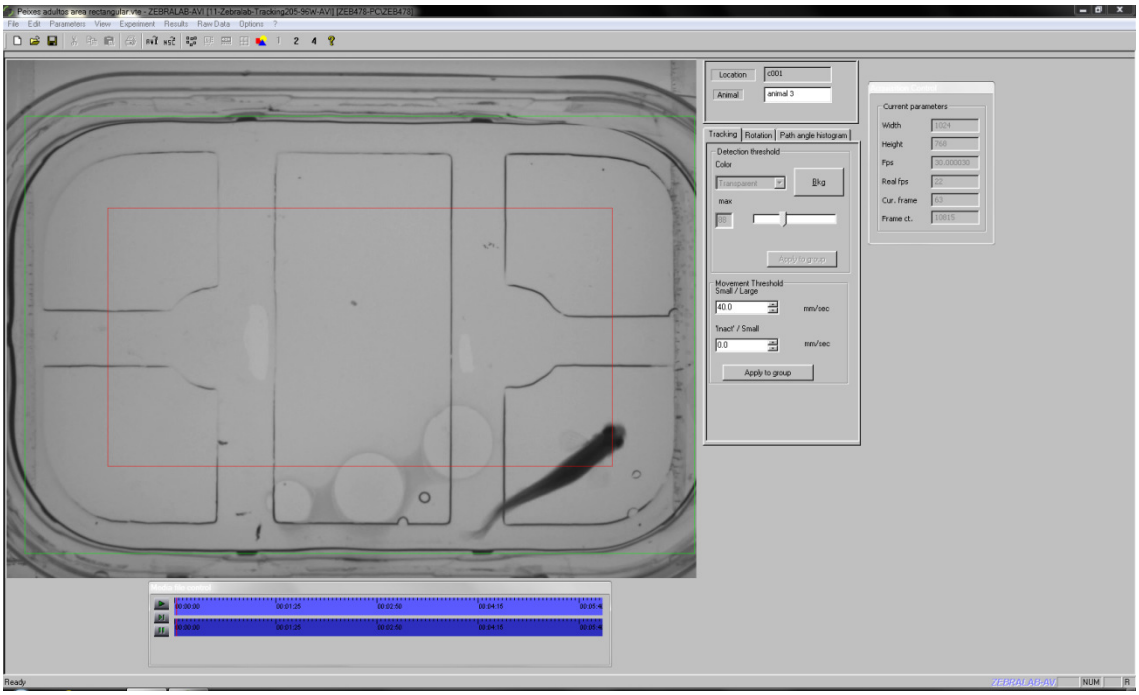

Figure S2 – Protocol used for the analysis of thigmotactic behavior, with the division of the aquarium in 2 areas (**Area 1 –** Outer zone; **Area 2 –** Inner zone).

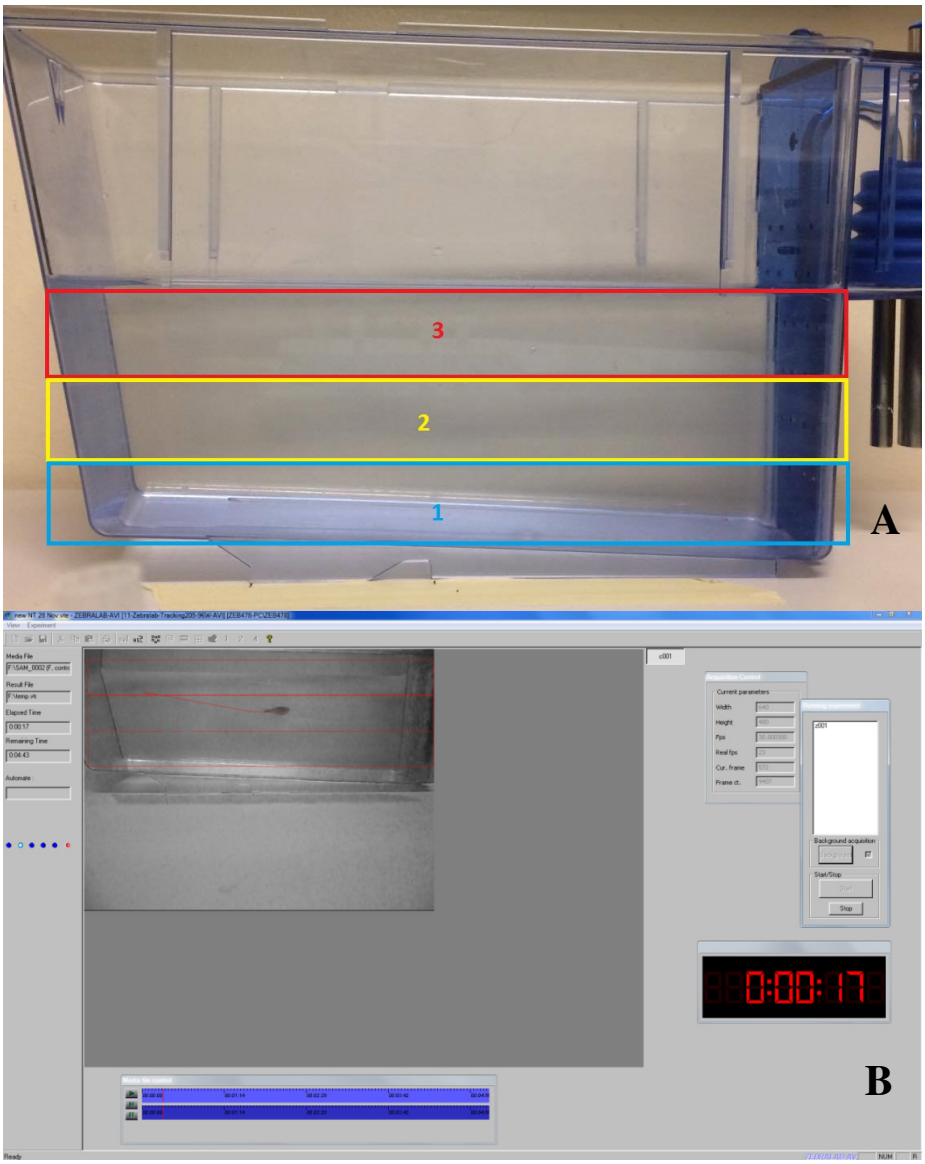

Figure S3 - Experimental assembly of the Exploratory Swimming test, with indication of the aquarium zones (Zone 1 – Bottom (blue), zone 2 – Middle (yellow) and zone 3 – Top (red)) (A) and print screen of the software ZebraLab® v3 Automated Behavioural Analysis used to aanalyse of the resulting videos (B).

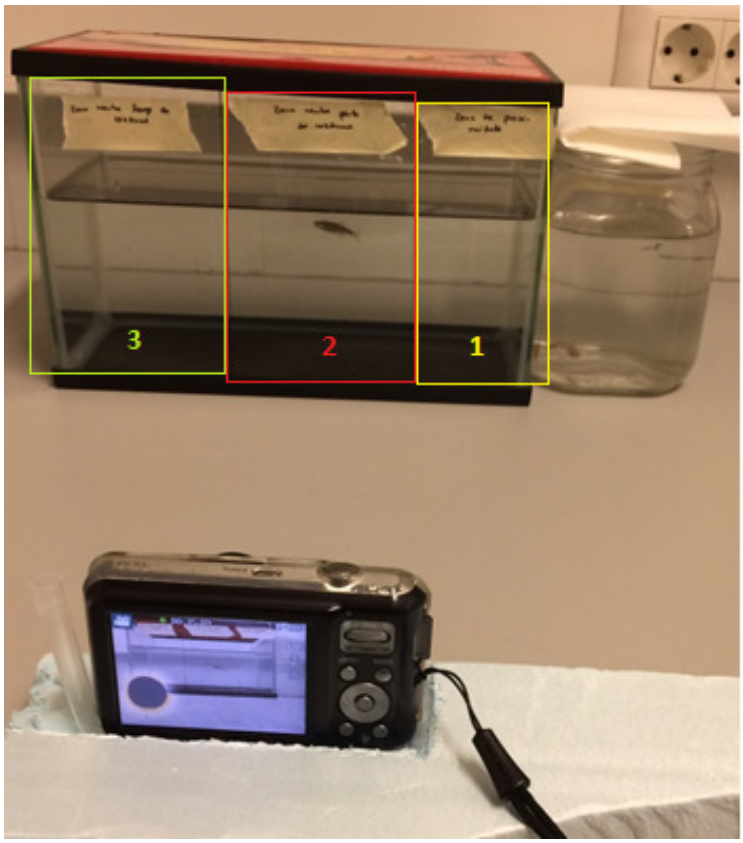

Figure S4 – Experimental assembly of the Shoaling test, with three zones: **Zone 1 -** Close proximity zone (the area closest to the shoal) (yellow color), **Zone 2 -** Neutral zone near the shoal (in the middle of the aquarium) (res color) and **Zone 3 -** Neutral zone away from the shoal (green color).

Figure S5 – Predator model used in the Predator Avoidance test.

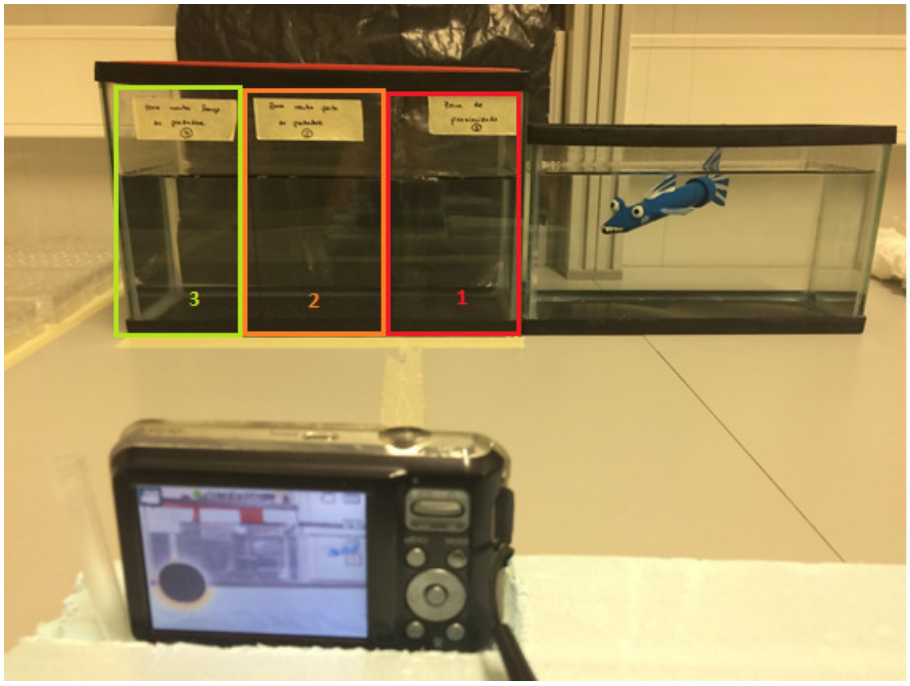

Figure S6 – Experimental assembly of the Predator Avoidance test, with three equal zones: **Zone 1 -** Close proximity zone (the area closest to the predator) (red color), **Zone 2 -** Neutral zone near the predator (orange color) and **Zone 3 -** Neutral zone away from the predator (green color).

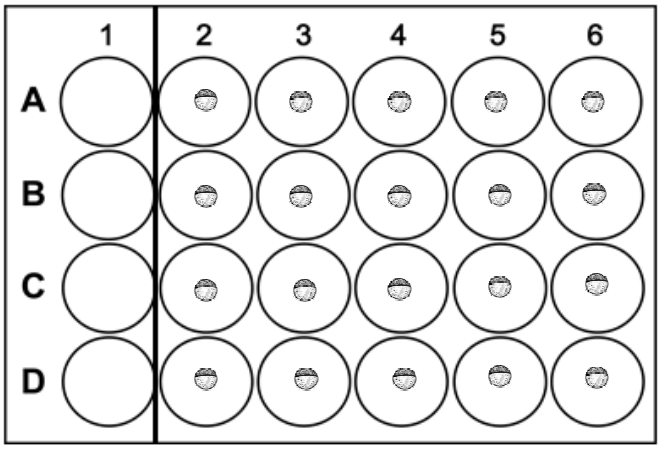

Figure S7 – Experimental design scheme used to observe the embryonic development of the F1 generation.

Chapter 3

Conclusion and Future Perspectives

Conclusion and Future Perspectives

Contamination of the aquatic environment by pesticides can induce effects at various levels, impairing the maintenance and survival of the organisms inserted in this environment. In the present work, we intend to evaluate the effects of the embryonic exposure to the sublethal concentrations of a contaminant, carbaryl, observing the consequences that these can produce in the zebrafish behavior when they are in the adult age.

The results showed the occurrence of significant behavioral changes. A decrease of the locomotor activity of the zebrafish exposed to carbaryl was observed, as well as a gradual decrease of the thigmotactic behavior in the outer zone, during the dark stimulus, and a reduction of the habituation to these same stimuli over the monitoring time. Pre-exposed zebrafish had less exploratory capacity, remaining longer at the bottom of the aquarium. Faced with this, the feeding behavior was also compromised, taking more time to feed. Pre-exposure to carbaryl also revealed that fish tend to remain closer to a shoal. However, the anti-predatory behavior and the reproduction and embryonic development of the F1 generation were not affected by this compound.

The behavior is influence by several biochemical and molecular pathways and physiological systems such as the endocrine, sensory, neuronal and metabolic system and all these changes mentioned during the present work may be due to the occurrence of disruptions in these processes.

According to the literature, carbamates inhibit the action of acetylcholinesterase, causing neurotoxic and muscular effects. It is important to understand and evaluate other pathways underlying these effects on behavior, as they could be the “key” to explain the changes that happen. These events may compromise the survival of a population, affecting several food chains. It is important to continue to analyze the effect that environmental contamination can have on the developmental programming of organisms by adapting other tests (used in other model organisms) and creating new one, aiming at the observation of long-term effects. It is also crucial to obtain valid behavioral tools for a better understanding of behavioral changes.

Thus, it is ecologically relevant to consider behavioral responses as a good indicator to be evaluated in ecotoxicity tests as they provide new findings of how embryonic exposure to very low amounts of a contaminant affects very sensitive endpoints at a later stage of development, with long-term effects.